

RULE 60

PRO
 10095/980400
 11/26/97



11/26/97
 J
 T
 Townsend and TOWNSEND and CREW LLP
 Embarcadero Center, 8th Floor
 Francisco, CA 94111-3834
 (415) 467-9600

ASSISTANT COMMISSIONER FOR PATENTS
 PATENT APPLICATION
 Washington, D.C. 20231

Sir:

This is a request under 37 CFR 1.60 for filing a
 Continuation Division
 of application No. 08/477,329, filed June 7, 1995,
 of (list each inventor) Andrzej Z. Sledziewski, Lillian A. Bell and Wayne R. Kindsvogel

for METHODS OF PRODUCING SECRETED RECEPTOR ANALOGS AND BIOLOGICALLY ACTIVE DIMERIZED POLYPEPTIDE FUSIONS

The application papers FILED HEREWITH (specification, claims, originally filed drawing(s) and oath or declaration) are a true copy of the prior application.

- Please amend the specification by inserting before the first line the sentence:
 --This is a [] Continuation Division of application No. 08/477,329, filed June 7, 1995 which is a continuation of USSN 08/180,195, filed January 11, 1994, issued as U.S. Patent No. 5,567,584; which is a file wrapper continuation of USSN 07/634,510, filed December 27, 1990; which is a continuation-in-part of USSN 07/347,291, filed May 2, 1989, issued as U.S. Patent No. 5,155,027; which is a continuation-in-part of USSN 07/146,877, filed January 22, 1988, now abandoned.
 the disclosure of which is incorporated by reference.--
- A preliminary amendment is enclosed.
 Formal drawings are enclosed.
 An Information Disclosure Statement under 37 CFR 1.97 is enclosed.
 Substitute Power of Attorney
 A verified statement to establish status under 37 CFR 1.9 and 37 CFR 1.27 [] is enclosed, or [] was filed in the above identified parent application.
 The prior application is assigned to ZymoGenetics, Inc.
 Please cancel claims 1-28

Claims as Filed, Less any Cancelled Claims

(Col. 1) (Col. 2)

FOR:	NO. FILED	NO. EXTRA
BASIC FEE		
TOTAL CLAIMS	30	-20= * 10
INDEP CLAIMS	4	-3= * 1
[] MULTIPLE DEPENDENT CLAIM PRESENTED		

* If the difference in Col. 1 is less than zero,
 enter "0" in Col. 2

SMALL ENTITY

RATE	FEES
	\$385
x11=	\$
x40=	\$
+130=	\$
TOTAL	\$

OTHER THAN A

SMALL ENTITY

RATE	FEES
	\$ 790
x22=	\$ 220
x80=	\$ 82
+260=	\$
TOTAL	\$1,092

Please charge Deposit Account No. 20-1430 as follows:

- Filing fee \$ 1,092
 Any additional fees associated with this paper or during the pendency of this application
 The issue fee set in 37 CFR 1.18 at or before mailing of the Notice of Allowance, pursuant to 37 CFR 1.311(b).

[] A check for \$ _____ is enclosed.
 2 extra copies of this sheet are enclosed.

Respectfully submitted,

TOWNSEND and TOWNSEND and CREW LLP

Jeffrey J. King
 Reg. No.: 38,515
 Attorneys for Applicant

Telephone: (415) 576-0200

I:\13952\53\2-1Div.TRN

RULE 60

TOWNSEND and TOWNSEND and CREW LLP
Two Embarcadero Center, 8th Floor
San Francisco, CA 94111-3834
(206) 467-9600

ASSISTANT COMMISSIONER FOR PATENTS
BOX PATENT APPLICATION
Washington, D.C. 20231

Sir:

This is a request under 37 CFR 1.60 for filing a

[] Continuation [X] Division
of application No. 08/477,329, filed June 7, 1995,
of (list each inventor) Andrzej Z. Sledziewski, Lillian A. Bell and Wayne R. Kindsvogel

Atty. Docket No. 13952A-005321

"Express Mail" Label No. EH314450612US

Date of Deposit 11-26-97

I hereby certify that this is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231

By Vin M. Goplen

for METHODS OF PRODUCING SECRETED RECEPTOR ANALOGS AND BIOLOGICALLY ACTIVE DIMERIZED POLYPEPTIDE FUSIONS

The application papers FILED HEREWITH (specification, claims, originally filed drawing(s) and oath or declaration) are a true copy of the prior application.

- [X] Please amend the specification by inserting before the first line the sentence:
 --This is a [] Continuation [X] Division of application No. 08/477,329, filed June 7, 1995 which is a continuation of USSN 08/180,195, filed January 11, 1994, issued as U.S. Patent No. 5,567,584; which is a file wrapper continuation of USSN 07/634,510, filed December 27, 1990; which is a continuation-in-part of USSN 07/347,291, filed May 2, 1989, issued as U.S. Patent No. 5,155,027; which is a continuation-in-part of USSN 07/146,877, filed January 22, 1988, now abandoned.
 the disclosure of which is incorporated by reference.--
- [X] A preliminary amendment is enclosed.
[X] Formal drawings are enclosed.
[X] An Information Disclosure Statement under 37 CFR 1.97 is enclosed.
[X] Substitute Power of Attorney
[X] A verified statement to establish status under 37 CFR 1.9 and 37 CFR 1.27 [] is enclosed, or [] was filed in the above identified parent application.
[X] The prior application is assigned to ZymoGenetics, Inc.
[X] Please cancel claims 1-28

Claims as Filed, Less any Cancelled Claims

(Col. 1)

(Col. 2)

SMALL ENTITY

FOR:	NO. FILED	NO. EXTRA
BASIC FEE		
TOTAL CLAIMS	30	-20= * 10
INDEP CLAIMS	4	-3= * 1
[] MULTIPLE DEPENDENT CLAIM PRESENTED		

* If the difference in Col. 1 is less than zero,
enter "0" in Col. 2

RATE	FEES
	\$385
x11=	\$
x40=	\$
+130=	\$
TOTAL	\$

OTHER THAN A
SMALL ENTITY

OR	RATE	FEES
OR		\$ 790
OR	x22=	\$ 220
OR	x80=	\$ 82
OR	+260=	\$
OR	TOTAL	\$1,092

Please charge Deposit Account No. 20-1430 as follows:

- [X] Filing fee \$ 1,092
[X] Any additional fees associated with this paper or during the pendency of this application
[X] The issue fee set in 37 CFR 1.18 at or before mailing of the Notice of Allowance, pursuant to 37 CFR 1.311(b).

Respectfully submitted,

[] A check for \$ _____ is enclosed.
2 extra copies of this sheet are enclosed.

TOWNSEND and TOWNSEND and CREW LLP

Jerry J. Kjeng
 Jeffrey J. Kjeng
 Reg. No.: 38,515
 Attorneys for Applicant

Telephone: (415) 576-0200

I:\13952\53\2-IDIV.TRN

I hereby certify that this correspondence is being deposited with the United States Postal Service as Express Mail #EH314450612US in an envelope addressed to: Box Patent Application, Assistant Commissioner for Patents, Washington, D. C. 20231 on 11-26-97

TOWNSEND and TOWNSEND and CREW
By Lin M. Sopler

PATENT

Attorney Docket No. 13952A-005321US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of)	
)	
Andrzej Z. Sledziewski,)	
Lillian A. Bell, and)	
Wayne R. Kindsvoegel)	
)	Examiner: Unassigned
Serial No.: Unassigned)	Parent Exr: C. Kaufman
)	
Parent Serial No. 08/477,329)	
)	Art Unit: Unassigned
Filed: Herewith)	Parent Art Unit: 1812
Parent Filed: June 7, 1995)	
)	
For: METHODS OF PRODUCING)	<u>PRELIMINARY AMENDMENT</u>
SECRETED RECEPTOR ANALOGS)	
AND BIOLOGICALLY ACTIVE)	
DIMERIZED POLYPEPTIDE)	
FUSIONS)	
)	

Box Patent Application
Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Prior to calculating the claims fee, please enter the following amendments in the above-identified application.

IN THE SPECIFICATION:

At page 1, please delete lines 7-11 setting forth cross-references to related applications and substitute therefor the following --This application is a divisional of U.S. Application Serial No. 08/477,329, filed June 7, 1995, which is a divisional of U.S. Application Serial No. 180,195, filed January 11, 1994, now Patent No. 5,567,584, which is a continuation of U.S. Application Serial No. 07/634,510 filed December 27, 1990, now abandoned, which is a continuation-in-

part of U.S. Application Serial No. 07/347,291, filed May 2, 1989, now Patent No. 5,155,027, which is a continuation-in-part of U.S. Application No. 07/146,877, filed January 22, 1988, now abandoned.

At page 6, line 11, please delete "encodes" and substitute therefor --encoded--.

At page 14, line 6, please delete "Figure 1" and insert therefor --Figures 1A-1D--.

At page 14, line 7, please delete "illustrates" and insert therefor --illustrate--.

At page 14, line 24, delete " μ promoter, μ enh; μ enhancer" and insert therefor -- μ promoter; μ enh, μ enhancer--.

At page 15, line 7, please delete "Figure 11 illustrates and insert therefor --Figures 11A-11D illustrate--".

At page 16, line 18, please delete "and/or are" and insert therefor --and/or is--.

At page 18, line 11, please change "doamin" to --domain--.

At page 18, line 25, please change "Figure 1" to --Figures 1A and 1B--.

At page 18, line 27, after "amino acid 531" please insert --(Figure 1B)--.

At page 18, line 29, please delete "Figure 11" and insert therefor --Figures 11A and 11B--.

At page 22, line 6, please delete "Figure 1" and insert therefor --Figures 1A and 1B--.

At page 22, line 9, please delete "Figure 1" and insert therefor --Figures 1A and 1B--.

At page 22, line 12, please delete "Figure 11" and insert therefor --Figures 11A and 11B--.

At page 22, line 14, please insert "a" between "of" and "smaller".

At page 25, line 23, please delete "joined is" and substitute therefor --is joined--.

At page 25, line 27, please delete "varable" and insert therefor --variable--.

At page 46, line 1, please delete "Figure 11" and insert therefor --Figures 11A-11D--.

At page 57, line 1, please delete "Figure 1" and insert therefor --Figure 1B--.

At page 57, line 2, please delete "Figure 1" and insert therefor --Figure 1B--.

At page 83, line 34, please delete "Figure 11" and insert therefor --Figure 11A--.

At page 84, line 22, please delete "Figure 11" and insert therefor --Figure 11B--.

At page 84, line 24, please delete "Figure 11" and insert therefor --Figure 11B--.

At page 88, line 7, please delete "was" and substitute therefor --were--.

IN THE CLAIMS:

Kindly amend the claims as follows:

Please cancel claims 1-28, without prejudice.

Please add the following new claims:

1 --29. A dimerized polypeptide fusion, comprising:
2 first and second polypeptide chains, wherein each of
3 said polypeptide chains comprises a non-immunoglobulin
4 polypeptide requiring dimerization for biological activity
5 joined to a dimerizing protein heterologous to said non-
6 immunoglobulin polypeptide.

1 30. The dimerized polypeptide fusion of claim 29
2 which is a homodimer.

1 31. The dimerized polypeptide fusion of claim 29
2 wherein the dimerizing protein of one of said polypeptide
3 chains comprises an immunoglobulin heavy chain constant
region.

1 32. The dimerized polypeptide fusion of claim 31
2 wherein the immunoglobulin heavy chain constant region is
3 joined to an immunoglobulin hinge region.

1 33. The dimerized polypeptide fusion of claim 31
2 wherein the immunoglobulin heavy chain constant region is
3 joined to an immunoglobulin variable region.

1 34. The dimerized polypeptide fusion of claim 33
2 wherein the immunoglobulin variable region is selected from
3 the group consisting of V_H , V_K , and V_λ .

1 35. The dimerized polypeptide fusion of claim 29
2 wherein the dimerizing protein one of said polypeptide chains
3 comprises an immunoglobulin heavy chain constant region domain
4 selected from the group consisting of C_{H1} , C_{H2} , C_{H3} , and C_{H4} of
5 a γ , α , ϵ , μ , or δ class immunoglobulin heavy chain.

1 36. The dimerized polypeptide fusion of claim 29
2 wherein the dimerizing protein one of said polypeptide chains
3 comprises an immunoglobulin light chain constant region.

1 37. A multimerized polypeptide fusion, comprising:

2 a non-immunoglobulin polypeptide requiring
3 multimerization for biological activity joined to an
4 immunoglobulin light chain constant region; and

5 an immunoglobulin heavy chain constant region domain
6 selected from the group consisting of C_H1, C_H2, C_H3, and C_H4.

1 38. The multimerized polypeptide fusion of claim 37
2 which is a tetramer comprising four polypeptide fusions each
3 having a non-immunoglobulin polypeptide joined to a
4 multimerizing protein.

1 39. The multimerized polypeptide fusion of claim 37
2 wherein the multimerizing protein comprises an immunoglobulin
3 heavy chain constant region.

1 40. The multimerized polypeptide fusion of claim 39
2 wherein the immunoglobulin heavy chain constant region is
3 joined to an immunoglobulin hinge region.

1 41. The multimerized polypeptide fusion of claim 39
2 wherein the immunoglobulin heavy chain constant region is
3 joined to an immunoglobulin variable region.

1 42. The multimerized polypeptide fusion of claim 41
2 wherein the immunoglobulin variable region is selected from
3 the group consisting of V_H, V_K, and V_L.

1 43. The multimerized polypeptide fusion of claim 37
2 wherein the multimerizing protein comprises an immunoglobulin
3 heavy chain constant region domain selected from the group
4 consisting of C_H1, C_H2, C_H3, and C_H4 of a γ , α , ϵ , μ , or δ class
5 immunoglobulin heavy chain.

1 44. The multimerized polypeptide fusion of claim 37
2 wherein the multimerizing protein comprises an immunoglobulin
3 light chain constant region.

1 45. A heteromultimeric polypeptide fusion,
2 comprising:

3 a first polypeptide fusion comprising a first non-
4 immunoglobulin polypeptide joined to a first multimerizing
5 protein heterologous to said first non-immunoglobulin
6 polypeptide and a second polypeptide fusion comprising a
7 second non-immunoglobulin polypeptide joined to a second
8 multimerizing protein heterologous to said second non-
9 immunoglobulin polypeptide.

1 46. The heteromultimeric polypeptide fusion of
2 claim 45 wherein the first and second non-immunoglobulin
3 polypeptides each comprise an amino acid sequence selected
4 from the group consisting of (A) the amino acid sequence of
5 Figures 1A-1D (Sequence ID Numbers 1 and 2), and (B) the amino
6 acid sequence of Figures 11A-11D (Sequence ID Numbers 35 and
7 36).

1 47. The heteromultimeric polypeptide fusion of
2 claim 45 wherein the first multimerizing protein is different
3 from the second multimerizing protein.

1 48. The heteromultimeric polypeptide fusion of
2 claim 47 wherein the first and second non-immunoglobulin
3 polypeptides are the same.

1 49. The heteromultimeric polypeptide fusion of
2 claim 45 wherein the first and second multimerizing proteins

3 each comprise an immunoglobulin heavy chain constant region or
4 an immunoglobulin light chain constant region.

1 50. The heteromultimeric polypeptide fusion of
2 claim 45 which comprises a first polypeptide fusion having a
3 first non-immunoglobulin polypeptide joined to a first
4 immunoglobulin constant region and a second polypeptide fusion
5 having a second non-immunoglobulin polypeptide fused to a
6 second immunoglobulin constant region different from the first
7 immunoglobulin constant region.

1 51. The heteromultimeric polypeptide fusion of
2 claim 50 wherein the first multimerizing protein comprises an
3 immunoglobulin heavy chain constant region and the second
4 multimerizing protein comprises an immunoglobulin light chain
5 constant region.

1 52. The heteromultimeric polypeptide fusion of
2 claim 49 wherein one of said multimerizing proteins comprises
3 an immunoglobulin heavy chain constant region joined to an
4 immunoglobulin hinge region.

1 53. The heteromultimeric polypeptide fusion of
2 claim 49 wherein one of said multimerizing proteins comprises
3 an immunoglobulin heavy chain constant region joined to an
4 immunoglobulin variable region.

1 54. The heteromultimeric polypeptide fusion of
2 claim 53 wherein the immunoglobulin variable region is
3 selected from the group consisting of V_H , V_K , and V_λ .

1 55. The heteromultimeric polypeptide fusion of
2 claim 45 wherein one of said multimerizing proteins comprises

3 an immunoglobulin heavy chain constant region domain selected
4 from the group consisting of C_H1, C_H2, C_H3, and C_H4 of a γ , α ,
5 ϵ , μ , or δ class immunoglobulin heavy chain.

1 56. The heteromultimeric polypeptide fusion of
2 claim 45 wherein said multimerized polypeptide fusion
3 comprises a first polypeptide fusion comprising a first
4 receptor or receptor domain requiring multimerization for
5 activity joined to a first immunoglobulin constant region and
6 a second polypeptide fusion comprising a second receptor or
7 receptor domain requiring multimerization for activity joined
8 to a second immunoglobulin constant region.

1 57. The heteromultimeric polypeptide fusion of
2 claim 56 wherein one of said receptor domains comprises a
3 ligand binding domain.

1 58. The heteromultimeric polypeptide fusion of
2 claim 45 which is a heterotetramer comprising four polypeptide
3 fusions each having a non-immunoglobulin polypeptide joined to
4 a multimerizing protein. --

REMARKS

By this amendment, claims 1-28 have been cancelled and new claims 29-58 have been added to more distinctly claim certain aspects of the invention. All of the amendments presented herein are fully supported by the specification, and no new matter has been added to the application. Specific support for new claims 29-58 is found throughout the specification, for example at pages 5-12, pages 23-25, pages 67-71, pages 71-73, pages 78-83 (Examples 13 and 14), and within the original claims as filed.

Formal Drawings

The Drawings have been amended to meet the separate numbering requirement of 37 CFR §1.84. The specification has also been amended to reflect these changes to Figures 1 and 11. Enclosed herewith are formal drawings incorporating these changes for the Draftperson's review and entry in the application.

Applicants believe that the claims presented herein for the Office's consideration are all in condition for allowance. Notice to that effect is requested. If for any reason, however, the Examiner feels that a telephone conference would expedite prosecution of the subject application, the Examiner is invited to telephone the undersigned at 206/467-9600.

Respectfully submitted,

Dated November 26, 1997 By: JJK
Jeffrey J. King
Reg. No. 38,515

TOWNSEND and TOWNSEND and CREW
Two Embarcadero Center, 8th Floor
San Francisco, CA 94111-3834
(206) 467-9600

I:\13952\53\2-1AMD.PRE

DECLARATION AND POWER OF ATTORNEY

As the below-named inventors, we declare that:

Our residences, post office addresses, and citizenships are as stated below under our names.

We have reviewed and understand the contents of the foregoing specification, including the claims, and we believe we are the original, first and joint inventors of the invention entitled "METHODS OF PRODUCING SECRETED RECEPTOR ANALOGS AND BIOLOGICALLY ACTIVE DIMERIZED POLYPEPTIDE FUSIONS," which is described and claimed in the specification and claims of patent application Serial No. 07/634,510, which we filed in the United States Patent and Trademark Office on December 27, 1990 (hereinafter referred to as "later C-I-P application"); and that this application in part discloses and claims subject matter disclosed in our earlier filed pending application entitled "METHODS OF PRODUCING SECRETED RECEPTOR ANALOGS AND BIOLOGICALLY ACTIVE PEPTIDE DIMERS," Serial No. 07/347,291, filed May 2, 1989 (hereinafter referred to as "earlier application").

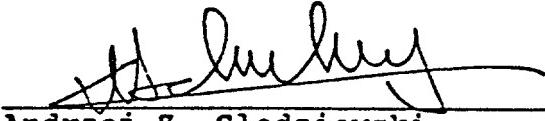
We hereby claim priority based on a foreign patent application filed in Europe on January 18, 1989, identified by Application No. 89100787.4

We acknowledge our duty to disclose information of which we are aware which is material to the examination of this application in accordance with 37 C.F.R. § 1.56(a), including material information which occurred between the filing date of said earlier filed pending application and the present.

We hereby appoint RICHARD W. SEED, Registration No. 16,557; ROBERT J. BAYNHAM, Registration No. 22,846; EDWARD W. BULCHIS, Registration No. 26,847; GEORGE C. RONDEAU, JR., Registration No. 28,893; DAVID H. DEITS, Registration No. 28,066; WILLIAM O. FERRON, JR., Registration No. 30,633; PAUL T. MEIKLEJOHN, Registration No. 26,569; DAVID J. MAKI, Registration No. 31,392; MICHAEL J. FOLISE, Registration No. 31,952; ROBERT M. STORWICK, Registration No. 30,112; RICHARD G. SHARKEY, Registration No. 32,629; GEORGE B. FOX,

Registration No. 31,510; DAVID V. CARLSON, Registration No. 31,153; MAURICE J. PIRIO, Registration No. 33,273; KARL R. HERMANNS, Registration No. 33,507; L. GRANT FOSTER, Registration No. 33,236; DAVID D. McMASTERS, Registration No. 33,963; and JOHN M. KELLY, Registration No. 33,920, composing the firm of SEED and BERRY, 6300 Columbia Center, Seattle, Washington 98104-7092, our attorneys to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. Please direct all telephone calls to David J. Maki at (206) 622-4900 and telecopies to (206) 682-6031.

We further declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that the making of willfully false statements and the like is punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and may jeopardize the validity of any patent issuing from this patent application.



Andrzej Z. Sledziewski

Date 03/01/71

Residence : City of Seattle, County of King
State of Washington

Citizenship : Poland

P.O. Address : 14543 - 30th Avenue N.E.
Seattle, Washington 98155

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants : Andrzej Z. Sledziewski, Lillian A. Bell, Wayne R. Kindsvogel
Serial No. : 08/477,329
Filed : June 7, 1995
For : METHODS OF PRODUCING SECRETED RECEPTOR ANALOGS
AND BIOLOGICALLY ACTIVE DIMERIZED POLYPEPTIDE FUSIONS

Examiner :
Art Unit : 1805
Docket : ZY1.P10C6

Assistant Commissioner of Patents

Washington, D.C. 20231

SUBSTITUTE POWER OF ATTORNEY

Sir:

I, Mark J. Murray, Vice President, Business Development of ZymoGenetics, Inc., a corporation of the State of Washington, having a place of business at 1201 Eastlake Avenue East, Seattle, WA 98102, hereby declare that ZymoGenetics, Inc. is the assignee of the above-identified application, which is a continuation of Serial No. 08/180,195, filed January 11, 1994, which is a continuation of Serial No. 07/634,510, filed December 27, 1990, which is a continuation in part of Serial No. 07/347,291, filed May 2, 1989 (U.S. Patent No. 5,155,027), which is a continuation in part of Serial No. 07/146,877, filed January 22, 1988. Documentary evidence of chain of title from the original owners to ZymoGenetics, Inc. has been filed with the United States patent and Trademark Office and has been recorded at Reel 5630, Frame 0638. I have reviewed the evidentiary documents referred to herein and it is certified that, to the best of my knowledge and belief, title is in ZymoGenetics, Inc.

ZymoGenetics, Inc., assignee of the entire interest in the above-mentioned application, hereby revokes all powers of attorney heretofore given in the above-referenced application and hereby appoints JEFFREY J. KING, Reg. No. 38,515; FRANK ABRAMONTE, Reg. No. 38,066; CHRIS E. SVENDSEN, Reg. No. 40,193; BETH A HAGEN, Reg. No. 37,032; and GEORGE A. CASHMAN, Reg. No. 26, 448, as the principal attorneys with full power of substitution, association and revocation to transact all business in

the Patent and Trademark Office connected therewith, and to all notices relating to the prosecution and issuance of this application. Please direct all future correspondence to:

Jeffrey J. King
STRATTON BALLEW PLLC
1218 Third Avenue, Suite 1313
Seattle, Washington 98101

and direct all telephone calls to Jeffrey J. King at (206) 682-1496 or facsimiles to (206) 682-0446.

Please change the attorney docket number to ZY1.P10C6.

I further represent that I have the authority to execute this document on behalf of ZymoGenetics, Inc. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that the making of willfully false statements and the like is punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and may jeopardize the validity of any patent issuing from this patent application.

7-15-96
Date


Mark J. Murray, Ph.D.
Vice President, Business Development

Client No.: 87-19C4

I hereby certify that this correspondence is being deposited with the United States Postal Service as Express Mail #EH314450612US in an envelope addressed to: Box Patent Application, Assist. Commissioner for Patents, Washington, D. C. 20231 on 11-26-97.

By Kim M. Yoplen

Patent
Attorney Docket No. 13952A-005321US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the application of)	
)	
Andrzej Z. Sledziewski,)	
Lillian A. Bell, and)	
Wayne R. Kindsvogel)	
Serial No.: Unassigned)	Examiner: Unassigned
)	Parent Exr: C. Kaufman
Parent Serial No. 08/477,329)	
)	Art Unit: Unassigned
Filed: Herewith)	Parent Art Unit: 1812
Parent Filed: June 7, 1995)	
For: METHODS OF PRODUCING)	TRANSMITTAL OF
SECRETED RECEPTOR ANALOGS)	FORMAL DRAWINGS
AND BIOLOGICALLY ACTIVE)	
DIMERIZED POLYPEPTIDE)	
FUSIONS)	
)	

Box Patent Application
Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

In regard to the above-identified application, Applicants submit eighteen (18) sheets of formal drawings to be made of record.

Respectfully submitted,

Dated: November 26, 1997

By: Jeffrey J. King
Jeffrey J. King
Reg. No. 38,515

TOWNSEND and TOWNSEND and CREW
Two Embarcadero Center, 8th Fl.
San Francisco, California 94111
(206) 467-9600
i:\13952\53\2-1DRAW.TRN

1	GGCCCTCAGCCCTGCTGCCAGCACGAGCCTGTGCTGCCCTGCCAACGCAGACAGCCAGACCCAGG	69
70	GCGGCCCTCTGGCGCTTGCTCCTCCGAAGGATGCTTGGGAGTGAGGCCAGCTGGCGCTCCTC	138
139	TCCCTACAGCAGCCCCCTTCCTCCATCCCTCTGTTCTCCTGAGCCTCAGGAGCCTGCCACAGTCCTG	207
208	CCTGTCTTCTACTCAGCTTACCCACTCTGGACCAGCAGTCTTCTGATAACTGGAGAGGGCAGT	276
277	AAGGAGGACTCCTGGAGGGGTGACTGTCCAGGCCTGAACTGTGCCACACCAAGCCATCAGCA	345
346	GCAAGGACACCATGCCGCTCCGGTGCATGCCAGCTGCCCTCAAAGGCAGCTGCTGTTGCTGT M R L P G A M P A L A L K G E L L L S	414 20
415	CTCTCCCTGTTACTCTGAAACCACAGATCTCTCAGGGCTGGTCGTACACCCCCGGGCCAGAGCTTG L L L L E P Q I S Q G L V V T P P G P E L V	483 43
484	TCCCTCAATGTCCTCCAGCACCTCGTTCTGACCTGCTCGGGTCAGCTCCGGTGTGAGGAAACGGATGT L N V S S T F V L T C S G S A P V V W E R M S	552 66
553	CCCAGGAGCCCCACAGGAATGCCAAGGCCAGGATGGCACCTCTCCAGCGTGCTCACACTGACCA Q E P P Q E M A K A Q D G T F S S V L T L T N	621 89
622	ACCTCACTGGCTAGACACGGAGAACTTTGACCCACAATGACTCCGTGGACTGGAGACCGATG L T G L D T G E Y F C T H N D S R G L E T D E	690 112
691	AGCGAACGGCTCTACATCTTGCCAGATCCCACCGTGGCTCCCTCCCTAATGATGCCGAGGAAC R K R L Y I F V P D P T V G F L P N D A E E L	759 135
760	TATTCACTTTCTCACGGAATAACTGAGATACCATTCCATGCCAGTAACAGACCCACAGCTGGGG F I F L T E I T E I T I P C R V T D P Q L V V	828 158
829	TGACACTGCAAGAGAAGGGACGTTGCACTGCCGTCCCTATGATCACCAACGTGGCTTTCTG T L H E K K G D V A L P V P Y D H Q R G F S G	897 181
898	GTATCTTGAGGACAGAAGCTACATCTGCAAAACCACCATGGGACAGGGAGGTGGATTCTGATGCC I F E D R S Y I C K T T I G D R E V D S D A Y	966 204
967	ACTATGTCTACAGACTCCAGGTGTCATCCATCACGTCTCTGTAACGCAGTGCAGACTGTGGCC Y V Y R L Q V S S I N V S V N A V Q T V V R Q	1035 227
1036	AGGGTGAGAACATCACCTCATGTGCATTGTGATGGGAATGAGGTGGTCAACTCGAGTGGACATACC G E N I T L M C I V I G N E V V N F E W T Y P	1104 250
1105	CCCGAAAGAAAGTGGCGGCTGGTGGAGCCGGTACTGACTTCCTCTGGATATGCCCTACACATCC R K E S G R L V E P V T D F L L D M P Y H I R	1173 273
1174	GCTCCATCCTGCACATCCCCAGTGCCAGTTAGAAGACTGGGGACCTACACCTGCAATGTGACGGAGA S I L H I P S A E L E D S G T Y T C N V T E S	1242 296
1243	GTGTGAATGACCATCAGGATGAAAGGCCATCAACATCACCGTGGTGAGAGCCGGTACGTGCCCTCC V N D H Q D E K A I N I T V V E S G Y V R L L	1311 319

Fig. 1A

1312 TGGGAGAGGTGGGCACACTACAATTGCTGAGCTGCATCGGAGCCGGACACTGCAGGTAGTGTTCGAGG 1380
 G E V G T L Q F A E L H R S R T L Q V V F E A 342
 1381 CCTACCCACCGCCCCTGCTCTGGTTCAAAGACAACCGCACCCCTGGCGACTCCAGCGCTGGCGAAA 1449
 Y P P P T V L W F K D N R T L G D S S A G E I 365
 1450 TCGCCCTGTCCACCGCAACGTGTCGGAGACCCGGTATGTGTCAGAGCTGACACTGGTCGCGTGAAGG 1518
 A L S T R N V S E T R Y V S E L T L V R V K V 388
 1519 TGGCAGAGGCTGCCACTACACCATGCGGGCCTTCCATGAGGATGCTGAGGTCCAGCTCCCTCCAGC 1587
 A E A G H Y T M' R A F H E D A E V Q L S F Q L 411
 1588 TACAGATCAATGTCCCTGTCCGAGTGCTGGAGCTAA GTGAGAGCCACCCCTGACAGTGGGAACAGACAG 1656
 Q I N V P V R V L E L S E S H P D S G E Q T V 434
 1657 TCCGCTGTCGTGGCCGGGGCATGCCCAAGCCAAACATCATCTGGTCTGCCTGCAGAGACCTAAAAGGT 1725
 R C R G R G M P Q P N I I W S A C R D L K R C 457
 1726 GTCCACGTGAGCTGCCGCCACGCTGCTGGGGAACAGTTCCGAAGAGGAGAGCCAGCTGGAGACTAACG 1794
 P R E L P P T L L G N S S E E E S Q L E T N V 480
 1795 TGACGTACTGGAGGAGGAGCAGGAGTTGAGGTGGTGAGCACACTGCCTGCAGCACGTGGATCGC 1863
 T Y W E E E Q E F E V V S T L R L Q H V D R P 503
 1864 CACTGTCGGTGCCTGCACGCTGCGAACGCTGTGGGCCAGGACACGCAGGAGGTACATCGTGGTGCAC 1932
 L S V R C T L R H A V G Q D T Q E V I V V P H 526
 1933 ACTCCCTGCCCTTAAGGTGGTGGTATCTCAGCCATCCTGGCCCTGGTGGTGCACCATCATCTCCC 2001
 S L P F K V V V I S A I L A L V V L T I I S L 549
 2002 TTATCATCCTCATCATGCTTGGCAGAAGAACGTTACGAGATCCGATGGAAGGTGATTGAGTCTG 2070
 I I L I M L W Q K K P R Y E I R W K V I E S V 572
 2071 TGAGCTCTGACGCCATGAGTACATCTACGTGGACCCATGCAGCTGCCCTATGACTCCACGTGGAGC 2139
 S S D G H E Y I Y V D P M Q L P Y D S T W E L 595
 2140 TGCCGGGGACCAGCTGTGCTGGACGCACCCCTGGCTCTGGGCCCTGGCAGGTGGTGGAGGCCA 2208
 P R D Q L V L G R T L G S G A F G Q V V E A T 618
 2209 CGGCTCATGCCCTGAGCATTCTCAGGCCACGATGAAAGTGGCCGTCAAGATGCTAAATCCACAGCCC 2277
 A H G L S H S Q A T M K V A V K M L K S T A R 641
 2278 GCAGCAGTGAGAACGCAAGCCCTATGCGAGCTGAAGATCATGAGTCACCTTGGCCCCACCTGAACG 2346
 S S E K Q A L M S E L K I M S H L G P H L N V 664
 2347 TGGTCAACCTGTTGGGGCTGCACCAAGGAGGACCCATCTATATCATCACTGAGTACTGCCCTACG 2415
 V N L L G A C T K G G P I Y I I T E Y C R Y G 687
 2416 GAGACCTGGTGGACTACCTGCACCGAACAAACACACCTTCCCTGCAGCACCACTCCGACAAGCGCC 2484
 D L V D Y L H R N K H T F L Q H H S D K R R P 710
 2485 CGCCCAAGCGCGGAGCTACAGCAATGCTGCCCCGTGGCTCCCCCTGCCAGCCATGTGTCCCTGA 2553
 P S A E L Y S N A L P V G L P L P S H V S L T 733

Fig. 1B

2554 CCGGGGAGAGCGACGGTGGCTACATGGACATGAGCAAGGACGAGTCGGTGGACTATGTGCCCATGCTGG 2622
 G E S D G G Y M D M S K D E S V D Y V P M L D 756
 2623 ACATGAAAGGAGACGTCAAATATGCAGACATCGAGTCCTCCAACATGGCCCTTACGATAACTACG 2691
 M K G D V K Y A D I E S S N Y M A P Y D N Y V 779
 2692 TTCCCTCTGCCCTGAGAGGACCTGCCGAGCAACTTTGATCAACGAGTCTCAGTGCTAAGCTACATGG 2760
 P S A P E R T C R A T L I N E S P V L S Y M D 802
 2761 ACCTCGTGGGCTTCAGCTACCAGGTGGCAATGGCATGGAGTTCTGGCCTCCAAGAACCTGCGTCCACA 2829
 L V G F S Y Q V A N G M E F L A S K N C V H R 825
 2830 GAGACCTGGCGGCTAGGAACGTGCTCATCTGTGAAGGCAAGCTGGTCAAGATCTGTGACTTGGCCTGG 2898
 D L A A R N V L I C E G K L V K I C D F G L A 848
 2899 CTCGAGACATCATCGGGACTCGAATTACATCTCAAAGGCAGCACCTTTGCCTTAAAGTGGATGG 2967
 R D I M R D S N Y I S K G S T F L P L K W M A 871
 2968 CTCCGGAGAGCATCTCAACAGCCTCTACACCACCTGAGCGACGTGGTCTGGGATCCTGCTCT 3036
 P E S I F N S L Y T T L S D V W S F G I L L W 894
 3037 GGGAGATCTTCACCTGGGTGGCACCCCTAACCGAGCTGCCATGAACGAGCAGTTCTACAATGCCA 3105
 E I F T L G G T P Y P E L P M N E Q F Y N A I 917
 3106 TCAAACGGGTTACCGCATGGCCCAGCCTGCCATGCCCTCCGACGGAGATCTATGAGATCATGAGAAGT 3174
 K R G Y R M A Q P A H A S D E I Y E I M Q K C 940
 3175 GCTGGGAAGAGAAGTTGAGATTGGCCCCCTCTCCAGCTGGTCTGCTCGAGAGAGACTGTTGG 3243
 W E E K F E I R P P F S Q L V L L E R L L G 963
 3244 GCGAAGGTTACAAAAGAAGTACCGAGGTGGATGAGGAGTTCTGAGGAGTGACCACCCAGCCATCC 3312
 E G Y K K K Y Q Q V D E E F L R S D H P A I L 986
 3313 TTGGTCCCAGGCCGCTGGCTGGTTCCATGGCCTCCGATCTCCCTGGACACCAGCTCCGTCTCT 3381
 R S Q A R L P G F H G L R S P L D T S S V L Y 1009
 3382 ATACTGCCGTGCAGCCAATGAGGTGACAACGACTATATCATCCCCCTGCTGACCCCAAACCCGAGG 3450
 T A V Q P N E G D N D Y I I P L P D P K P E V 1032
 3451 TTGCTGACGAGGGCCCCTGGAGGGTTCCCCCAGCCTAGCCAGCTCCACCCCTGAATGAAGTCACACCT 3519
 A D E G P L E G S P S L A S S T L N E V N T S 1055
 3520 CCTCAACCCTCTCTGTGACAGCCCCCTGGAGCCCCAGGACGAACCAGGCCAGAGCCCCAGCTTGAGC 3588
 S T I S C D S P L E P Q D E P E P E P Q L E L 1078
 3589 TCCAGGTGGAGCCGGAGCCAGAGCTGGAACAGTTGCCGATTGGGGTGCCCTGCCCTGGGGAG 3657
 Q V E P E P E L E Q L P D S G C P A P R A E A 1101
 3658 CAGAGGATAGCTTCTGTAGGGGCTGGCCCTACCCCTGCCCTGCCCTGAAGCTCCCCCTGCCAGCAC 3726
 E D S F L . 1106
 3727 CCAGCATCTCCTGGCCTGGCCTGACCGGGCTTCTGTCAAGCCAGGCTGCCCTATCAGCTGTCCCTTC 3795

Fig. 1C

3796 TGGAAGCTTCTGCTCCTGACGTGGTGCCTGGCCAAACCCCTGGGCTGGCTTAGGAGGCAAGAAAATG 3864
3865 AGGGGCCGTGACCAGCCCTCTGCCTCCAGGGAGGCCACTGACTCTGAGCCAGGGTTCCCCCAGGGAAC 3933
3934 TCAGTTTCCCATAATGTAAGATGGAAAGTTAGGCTTGATGACCCAGAAATCTAGGATTCTCTCCCTGGC 4002
4003 TGACACGGTGGGAGACCGAATCCCTCCCTGGGAAGATTCTGGAGTTACTGAGGTGGTAAATTAAACAT 4071
4072 TTTTCTGTTAGCCAGCTACCCCTCAAGGAATCATAGCTCTCTCGCACTTTTATCCACCCAGGA 4140
4141 GCTAGGGAAGAGACCCCTAGCCTCCCTGGCTGCTGGCTGAGCTAGGGCCTAGCTGAGCAGTGGTGCCTC 4209
4210 ATCCAGAAGAAAGCCAGTCCTCCCTATGATGCCAGTCCCTGCCGTTCCCTGGCCCGAGCTGGTCTGGG 4278
4279 GCCATTAGGCAGCCTAATTAAATGCTGGAGGCTGAGCCAAGTACAGGACACCCCCAGCCTGCAGCCCTG 4347
4348 CCCAGGGCACTTGGAGCACACGCGACCCATAGCAAGTGCCTGTGTCCTGTCCCTCAGGCCATCAGTCC 4416
4417 TGGGGCTTTCTTTATCACCCCTCAGCTTAATCCATCCACCAGAGTCTAGAAGGCCAGACGGCCCCG 4485
4486 CATCTGTGATGAGAATGAAATGTGCCAGTGTGGAGTGGCACGTGTGTGCCAGTATATGCCCTGG 4554
4555 CTCTGCATTGGACCTGCTATGAGGCTTGGAGGAATCCCTCACCCCTCTGGGCCTCAGTTCCCTTC 4623
4624 AAAAAATGAATAAGTCGGACTTATTAACACTCTGAGTGCCTGCCAGCACTAACATTCTAGAGTATTCCAG 4692
4693 GTGGTGCACATTGTCCAGATGAAGCAAGGCCATACCCCTAAACTTCATCCTGGGGTCAGCTGGCTC 4761
4762 CTGGGAGATTCCAGATCACACATCACACTCTGGGACTCAGGAACCATGCCCTTCCCCAGGCCAG 4830
4831 CAAGTCTCAAGAACACAGCTGCACAGGCCCTGACTTAGAGTGACAGCCGTCTGGAAAGCCCCAAG 4899
4900 CAGCTGCCCAAGGGACATGGGAAGACCACGGACCTTTCACTACCCACGATGACCTCCGGGGTATC 4968
4969 CTGGGCAAAAGGGACAAGAGGGCAAATGAGATCACCTCCTGCAGCCCACCACTCCAGCACCTGTGCCG 5037
5038 AGGTCTCGTCAAGACAGAATGGACAGTGAGGACAGTTATGTCTTGTAAAAGACAAGAAGCTTCAGAT 5106
5107 GGTACCCCAAGAAGGATGTGAGAGGTGGCTGCTTGGAGTTGCCCTCACCCACAGCTGCCCAT 5175
5176 CCCTGAGGCATGCGCTCCATGGGGTATGGTTTGTCACTGCCAGACCTAGCAGTGACATCTCATTGT 5244
5245 CCCCAGCCCAAGTGGCATTGGAGGTGCCAGGGAGTCAGGGTTGTAGCCAAGACGCCCGCACGGGA 5313
5314 GGGTTGGGAAGGGGTGCAGGAAGCTCAACCCCTCTGGCACCAACCCCTGCATTGCAGGTTGGCACCTT 5382
5383 ACTTCCCTGGGATCCCCAGAGTTGGTCCAAGGAGGGAGGTGGTTCTCAATACGGTACCAAAAGATATA 5451
5452 ATCACCTAGGTTACAATATTTAGGACTCACGTTAACTCACATTATAACAGCAGAAATGCTATT 5520
5521 GTATGCTGTTAAGTTTTCTATCTGTGTACTTTTTAAGGGAAAGATTT 5572

Fig. 1D

Figure 2

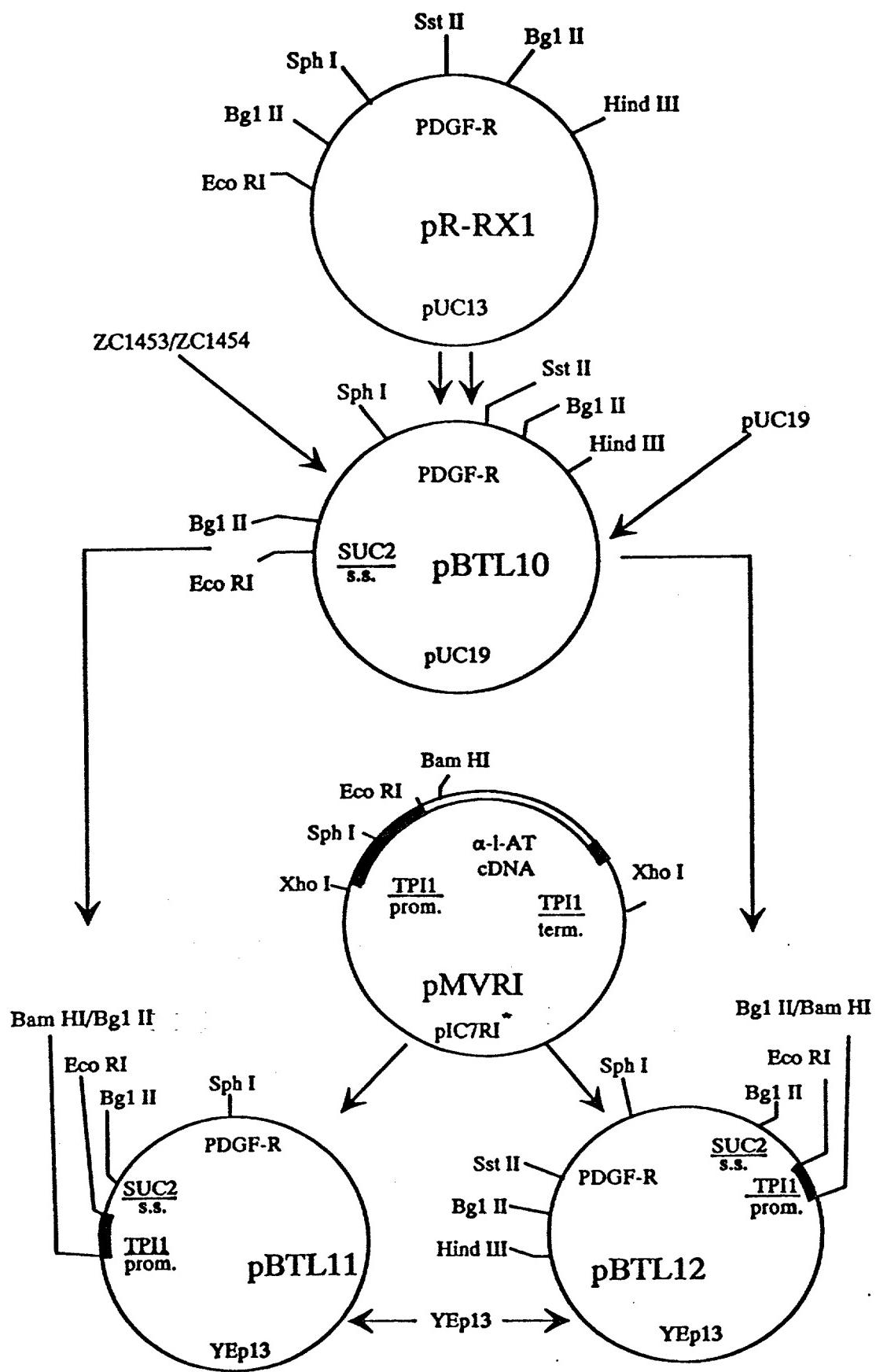


Figure 3

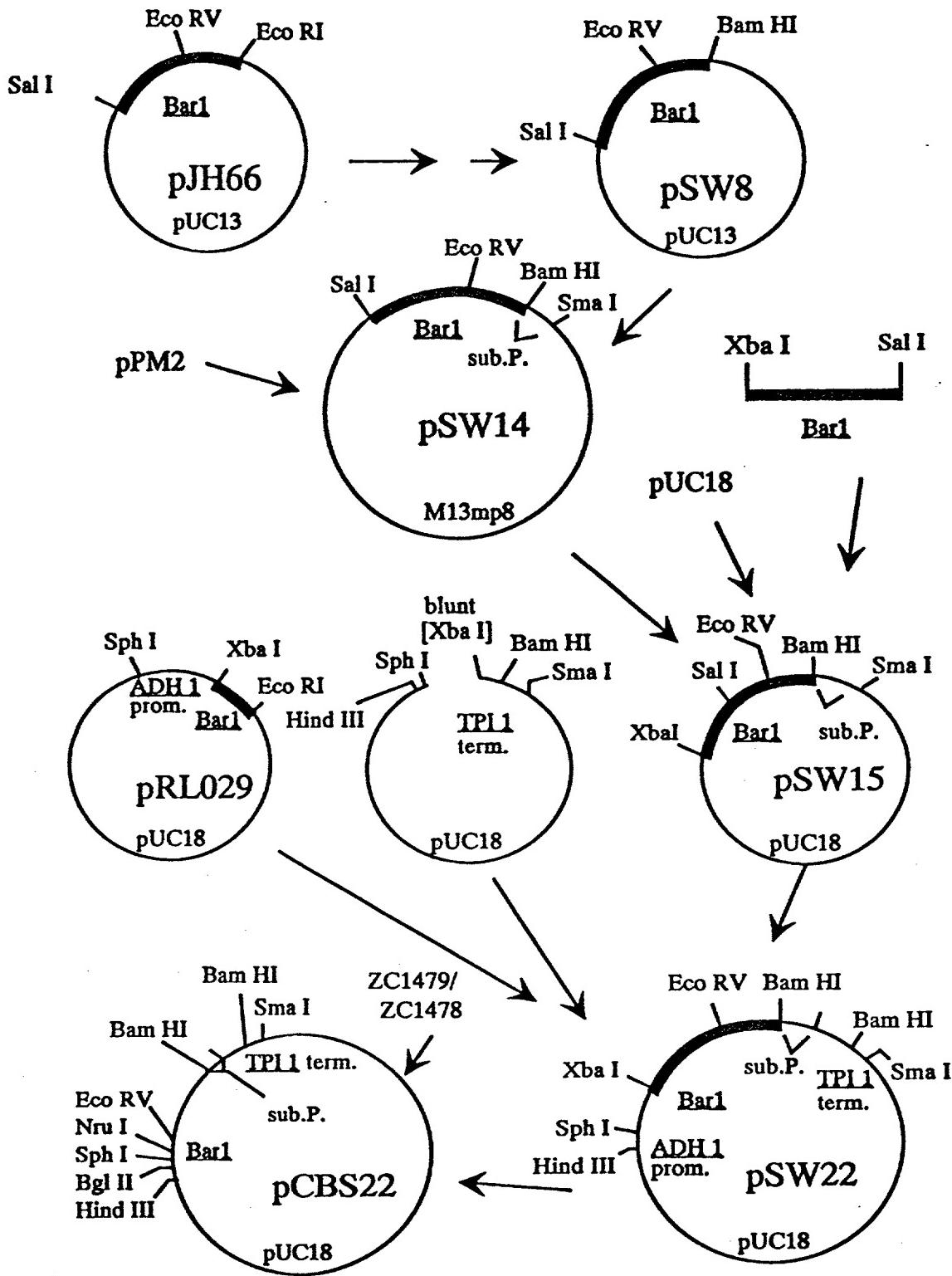


Figure 4

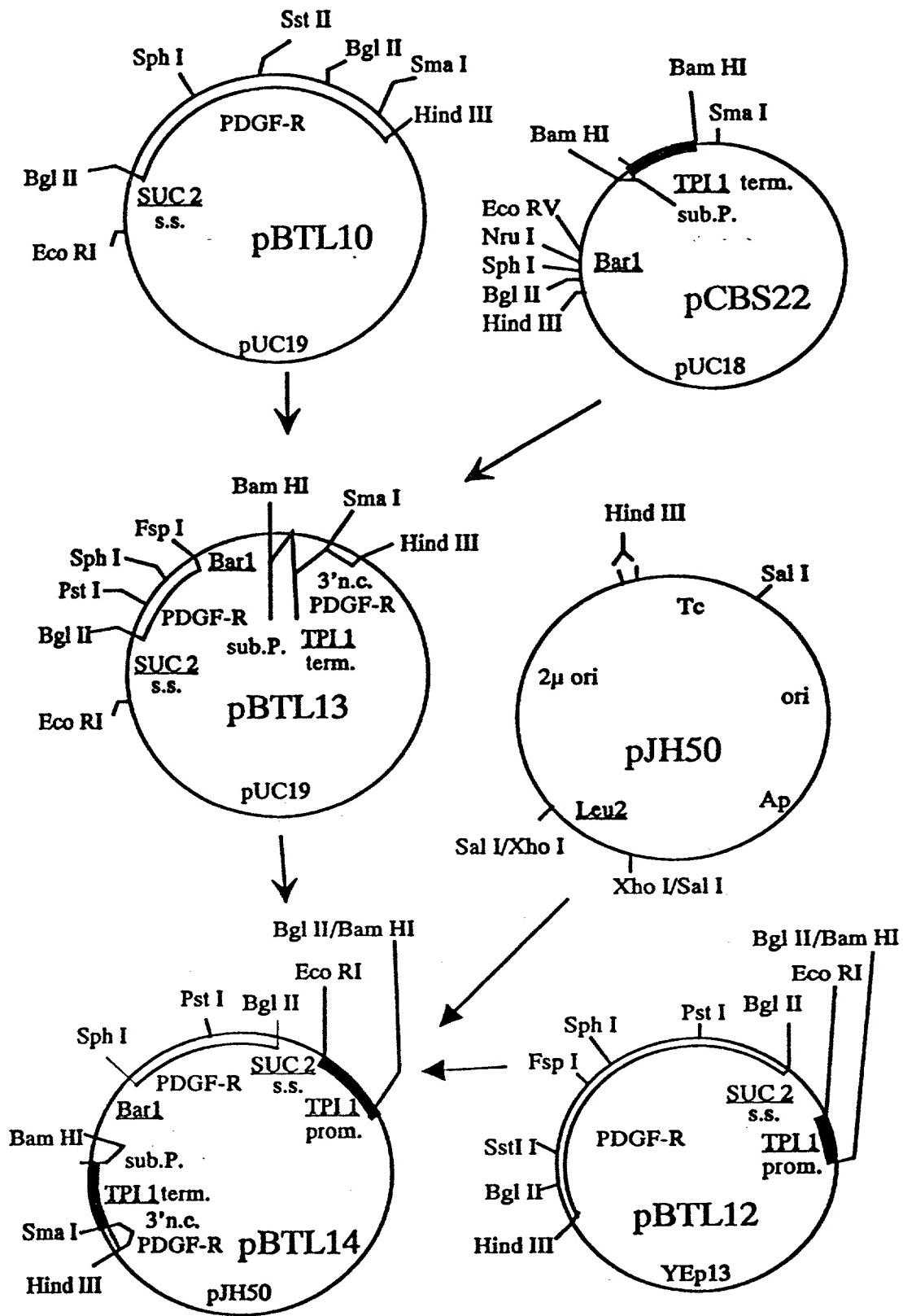


Figure 5

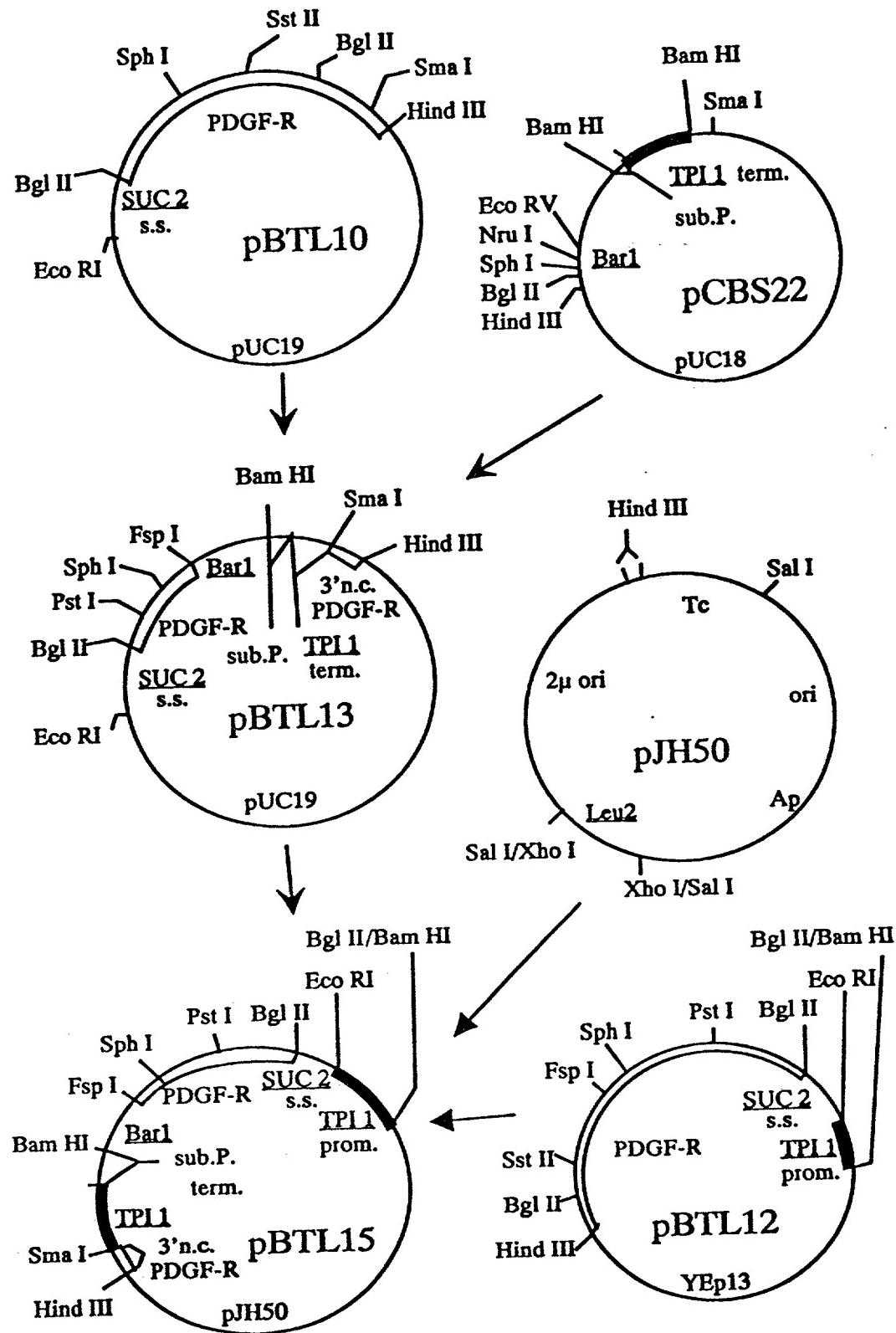


Figure 6

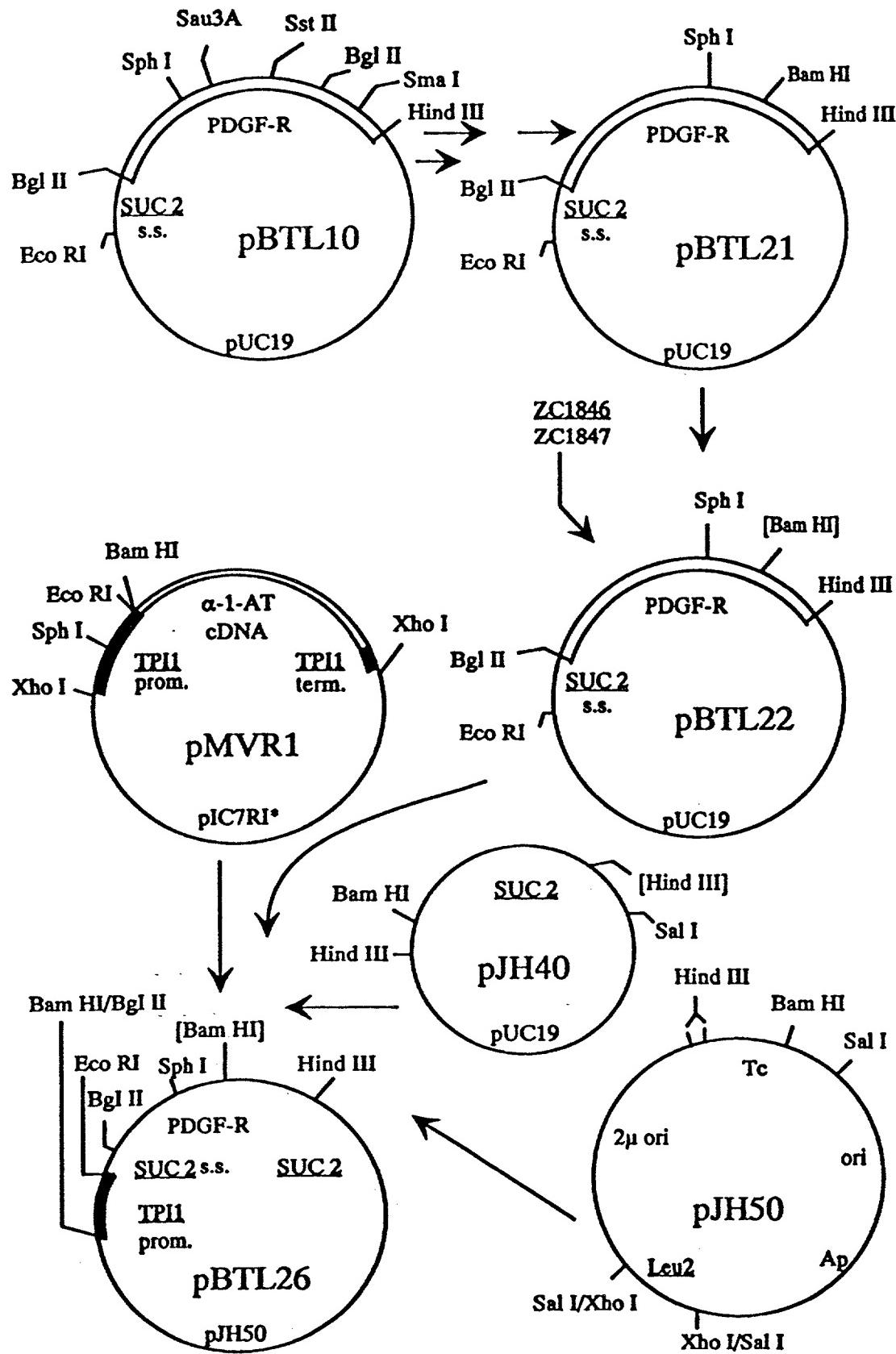


Figure 7

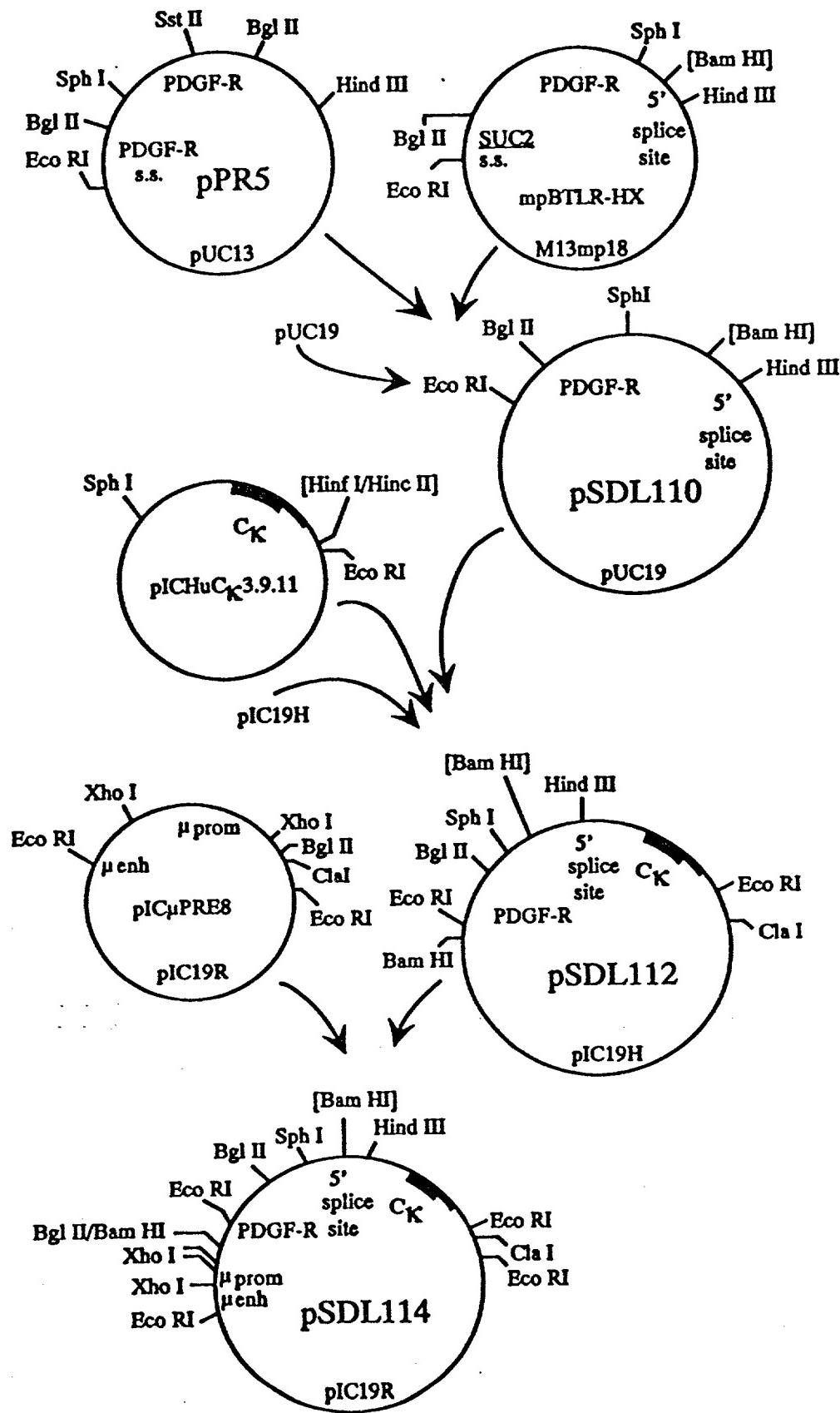


Figure 8

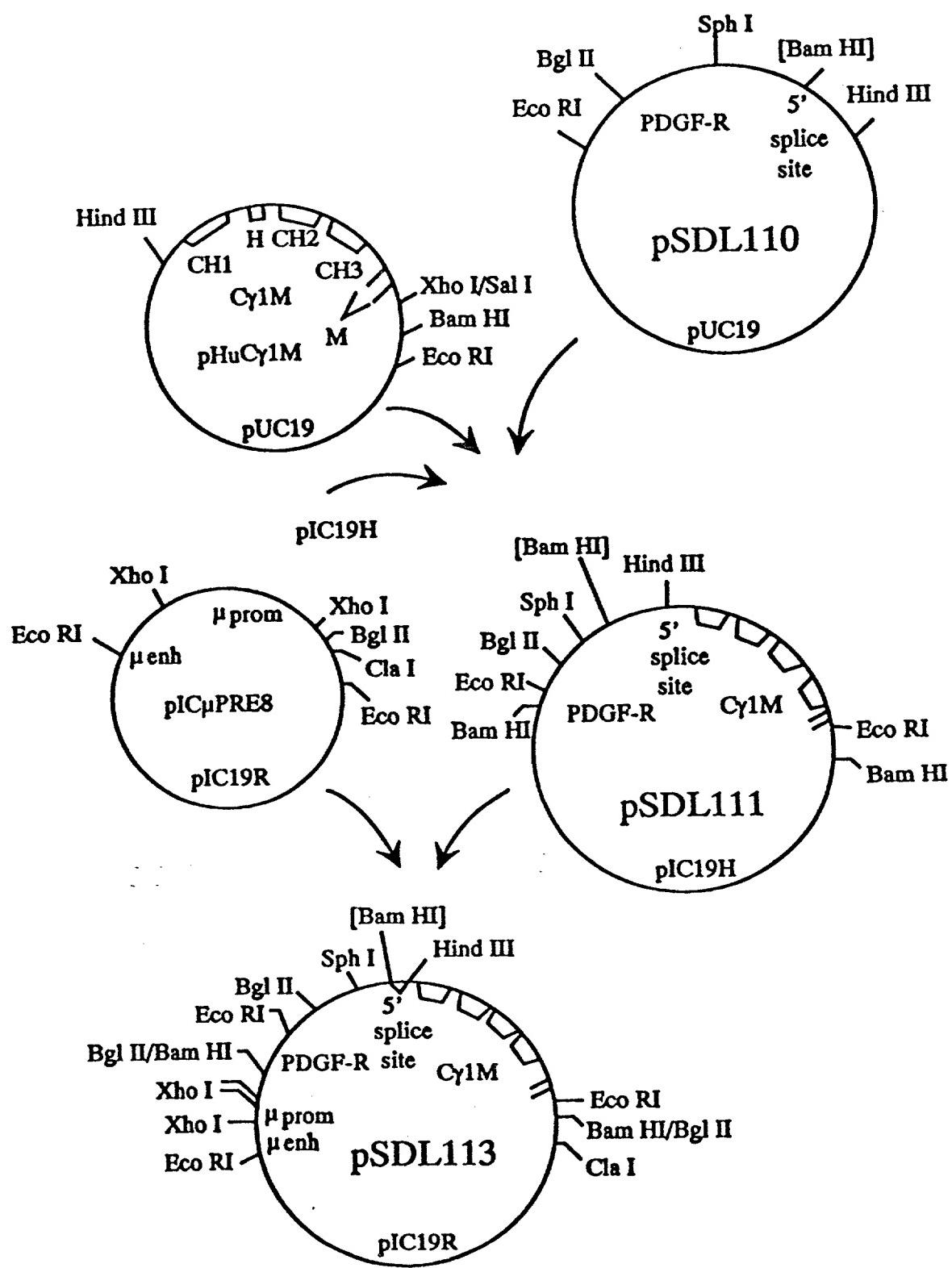


Figure 9

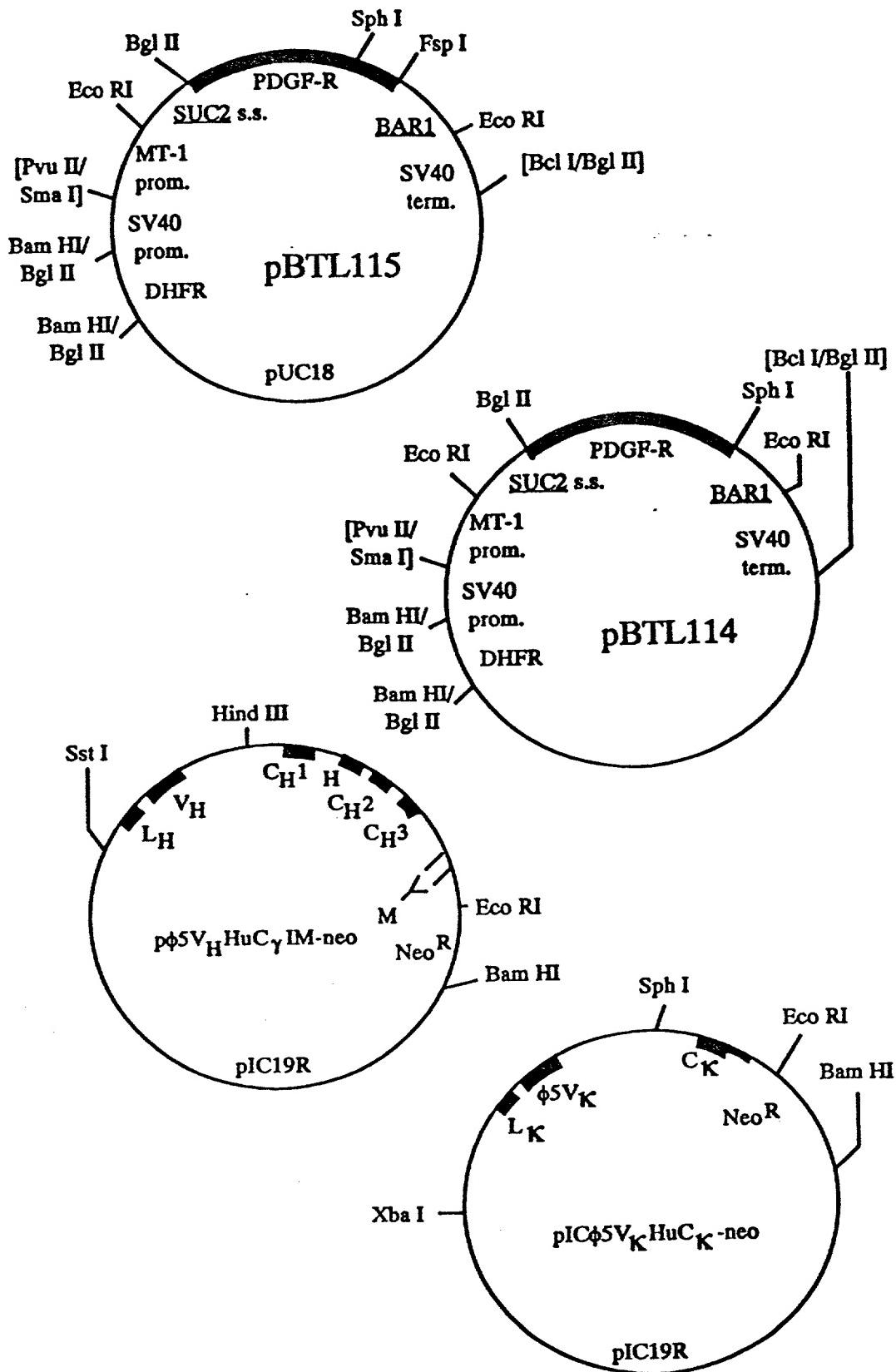


Figure 10

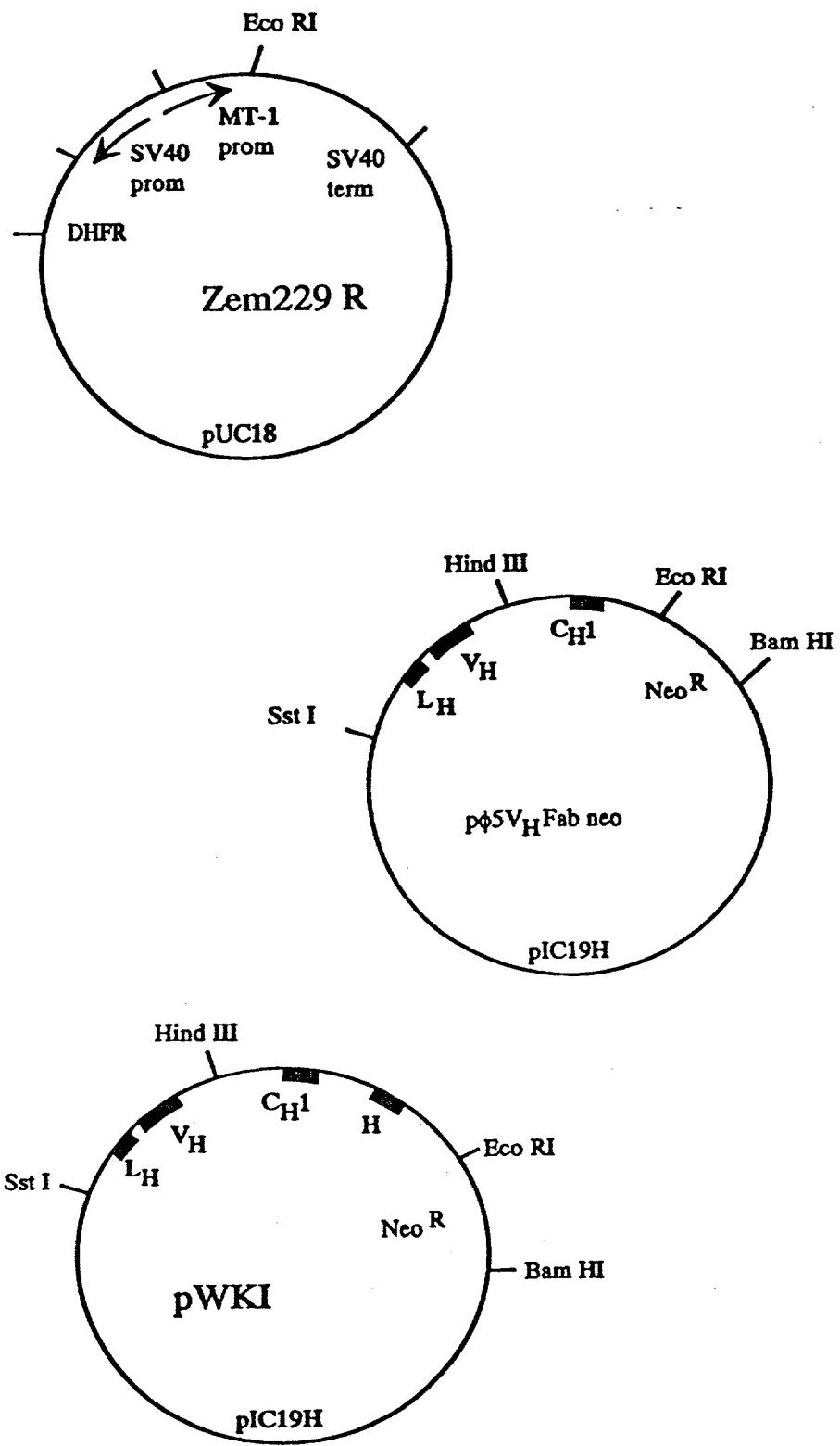


Figure 11A

1 GCCCTGGGACGGACCCTGGCGGCCGCGCAGCGGGGGACGCGTTGGGACGTGGTGGCCAGCGCCT
70 TCCTGCAGACCCCACAGGGAAAGTACTCCCTTGACCTCCGGGGAGCTGCGACCAGGTTACGTTGCTGG
139 TGGAAAAGTGACAATTCTAGGAAAAGAGCTAAAAGCCGGATCGGTGACCGAAAGTTCCCAGAGCTATG
M
1
208 GGGACTTCCCATCCGGCGTTCTGGTCTTAGGCTGTCTCTCACAGGGCTGAGCCTAACCTCTGCCAG
G T S H P A F L V L G C L L T G L S L I L C Q
277 CTTTCATTACCCCTATCCTTCAAATGAAAAGGTTGTGCAGCTGAATTACATCCTTTCTCTG
L S L P S I L P N E N E K V V Q L N S S F S L
346 AGATGCTTGGGGAGAGTGAAAGTGAGCTGGCAGTACCCCCATGTCTGAAGAAGAGAGCTCCGATGTGGAA
R C F G E S E V S W Q Y P M S E E E S S D V E
415 ATCAGAAATGAAGAAAACAACAGCGGCCCTTTGTGACGGTCTTGGAAAGTGAGCAGTGCCTCGGCGGCC
I R N E E N N S G L F V T V L E V S S A S A A
484 CACACAGGGTTGTACACTTGCTATTACAACCAACTCAGACAGAAGAGAAATGAGCTTGAAGGCAGGCAC
H T G L Y T C Y Y N H T Q T E E N E L E G R H
553 ATTACATCTATGTGCCAGACCCAGATGTAGCCTTGTACCTCTAGGAATGACGGATTATTTAGTCATC
I Y I Y V P D P D V A F V P L G M T D Y L V I
622 GTGGAGGATGATGATTCTGCCATTATAACCTTGTGCACAACTGATCCGAGACTCCTGTAACCTTACAC
V E D D D S A I I P C R T T D P E T P V T L H
691 AACAGTGAGGGGGTGGTACCTGCCCTACGACAGCAGACAGGGCTTAAATGGGACCTTCACTGTAGGG
N S E G V V P A S Y D S R Q G F N G T F T V G
760 CCCTATATCTGTGAGGCCACCGTCAAAGGAAAGAAGTCCAGACCATCCATTAAATGTTATGCTTAA
P Y I C E A T V K G K K F Q T I P F N V Y A L
829 AAAGCAACATCAGAGCTGGATCTAGAAATGGAAGCTTAAACCGTGTATAAGTCAGGGAAACGATT
K A T S E L D L E M E A L K T V Y K S G E T I
898 GTGGTCACCTGTGCTTTAACATGAGGTGGTGCACCTCAATGGACTTACCCGGAGAAGTGA
V V T C A V F N N E V V D L Q W T Y P G E V K

Figure 11B

967 GGC₁AAGG₂CATCACAAT₃ACTGG₄AAGAA₅ATCAA₆AGTCCC₇CATCC₈ATCAA₉ATTGG₁₀GTACACT₁₁TTGACGG₁₂TC
G K G I T I L E E I K V P S I K L V Y T L T V

1036 CCCGAGGCCACGGT₁GAAAGACAGTGGAGATTACGAATGTGCTGCCGCCAGG₂CTACCAGGGAGGTCAAA
P E A T V K D S G D Y E C A A R Q A T R E V K

1105 GAAATGAAGAAAGTCACTATTCTGTCATGAGAAAGGTT₁CATTGAAATCAAACCCACCTTCAGCCAG
E M K K V T I S V H E K G F I E I K P T F S Q

1174 TTGGAAAGCTGTCAACCTGCATGAAGTCAAACATT₁TTGTTGAGGGTGC₂GGGC₃CTACCCACCTCCCAGG
L E A V N L H E V K H F V V E V R A Y P P P R

1243 ATATCCTGGCTGAAAACAATCTGACTCTGATTGAAAATCTCACTGAGATCACC₁ACTGATGTGGAAAAG
I S W L K N N L T L I E N L T E I T T D V E K

1312 ATTCA₁GGAAATAAGGTATCGAAGC₂AAATTAAAGCTGATCCGTGCTAAGGAAGAAGACAGTGGCCATTAT
I Q E I R Y R S K L K L I R A K E E D S G H Y

1381 ACTATTGTAGCTCAAATGAAGATGCTGTGAAGAGCTATACTTTGA₁ACTGTTA₂ACTCAAGT₃TCTCA
T I V A Q N E D A V K S Y T F E L L L T Q V P S

1450 TCCATTCTGGACTTGGTCGATGATCACCATGGCTCACTGGGGACAGACGGTGAGGTGCACAGCTGAA
S I L D L V D D H H G S T G G Q T V R C T A E

1519 GGCACGCCGCTCCTGATATTGAGTGGATGATATGCAAAGATATTAAAGAA₁ATGTAATAATGAAACTTCC
G T P L P D I E W M I C K D I K K C N N E T S

1588 TGGACTATTGGCCAACAATGTCTCAAACATCATCACGGAGATCCACTCCCAGACAGGAGTACCGTG
W T I L A N N V S N I I T E I H S R D R S T V

1657 GAGGGCCGTGTGACTTCGCCAAAGTGGAGGAGACC₁TGCCGTGCGATGCC₂TGGCTAAGAATCTCCTT
E G R V T F A K V E E T I A V R C L A K N L L

1726 GGAGCTGAGAACCGAGAGCTGAAGCTGGTGCTCCACCC₁TGCGTTCTGA₂ACTCACGGTGGCTGCTGCA
G A E N R E L K L V A P T L R S E L T V A A A

1795 GTCTGGTGTGTTGGT₁GATTGTGATCATCTCACTTATTGCTGTTGTCA₂TTGGAAACAGAAACCG
V L V L L V I V I I S L I V L V V I W K Q K P

• — • — • — • — • —

Figure 11C

1864 AGGTATGAAATTGCTGGAGGGTCAATTGAATCAATCAGCCCGATGGACATGAATATATTATGTGGAC
R Y E I R W R V I E S I S P D G H E Y I Y V D

1933 CCGATGCAGCTGCCATTACTCAAGATGGAGTTCCAAGAGATGGACTAGTGCTTGGTCGGTCTTG
P M Q L P Y D S R W E F P R D G L V L G R V L

2002 GGGTCTGGAGCGTTGGGAAGGTGGTTGAAGGAACAGCCTATGGATTAAAGCCGGTCCCACCTGTCAATG
G S G A F G K V V E G T A Y G L S R S Q P V M

2071 AAAGTTGCAGTGAAGATGCTAAAACCACGGCCAGATCCAGTAAAAACAAGCTCTCATGTCTGAAC TG
K V A V K M L K P T A R S S E K Q A L M S E L

2140 AAGATAATGACTCACCTGGGCCACATTGAACATTGTAACATTGCTGGAGCCTGCACCAAGTCAGGC
K I M T H L G P H L N I V N L L G A C T K S G

2209 CCCATTACATCATCACAGAGTATTGCTCTATGGAGATTGGTCAACTATTGCATAAGAATAGGGAT
P I Y I I T E Y C F Y G D L V N Y L H K N R D

2278 AGCTTCCTGAGCCACCACCCAGAGAACGCAAAGAAAGAGCTGGATATCTTGGATTGAACCTGCTGAT
S F L S H H P E K P K K E L D I F G L N P A D

2347 GAAAGCACACGGAGCTATGTTATTTATCTTGAACATGGACTACATGGACATGAAGCAGGCT
E S T R S Y V I L S F E N N G D Y M D M K Q A

2416 GATACTACACAGTATGCCCCATGCTAGAAAGGAAAGAGGTTCTAAATATTCCGACATCCAGAGATCA
D T T Q Y V P M L E R K E V S K Y S D I Q R S

2485 CTCTATGATCGTCCAGCCTCATATAAGAAGAAATCTATGTTAGACTCAGAAGTCAAAACCTCCTTCA
L Y D R P A S Y K K K S M L D S E V K N L L S

2554 GATGATAACTCAGAAGGCCATTACTTATTGGATTGTTGAGCTTACCTATCAAGTTGCCGAGGAATG
D D N S E G L T L L D L L S F T Y Q V A R G M

2623 GAGTTTTGGCTTCAAAAAATTGTGTCACCGTGATCTGGCTGCTCGAACGTCTCTGGCACAGGA
E F L A S K N C V H R D L A A R N V L L A Q G

2692 AAAATTGTGAAGATCTGTGACTTGGCCTGGCCAGAGACATCATGCATGATTGAACTATGTGTCGAAA
K I V K I C D F G L A R D I M H D S N Y V S K

2761 GGCAGTACCTTCTGCCGTGAAGTGGATGGCTCTGAGAGCATCTTGACAACCTCTACACCACACTG
G S T F L P V K W M A P E S I F D N L Y T T L

• • • • •

Figure 11D

2830 AGTGATGTCTGGTCTTATGGCATTCTGCTCTGGAGATCTTTCCCTGGTGGCACCCCTAACCGGC
S D V W S Y G I L L W E I F S L G G T P Y P G
2899 ATGATGGTGGATTCTACTTTCTACAATAAGATCAAGAGTGGTACCGATGGCCAAGCCTGACCACGCT
M M V D S T F Y N K I K S G Y R M A K P D H A
2968 ACCAGTGAAGTCTACGAGATCATGGTAAATGCTGGAACAGTGAGCCGGAGAAGAGACCCCTCTTAC
T S E V Y E I M V K C W N S E P E K R P S F Y
3037 CACCTGAGTGAGATTGTGGAGAATCTGCTGCCTGGACAATATAAAAAGAGTTATGAAAAAAATTACCTG
H L S E I V E N L L P G Q Y K K S Y E K I H L
3106 GACTTCCTGAAGAGTGACCATCCTGCTGTGGCACGCATGCGTGTGGACTCAGACAATGCATACTGGT
D F L K S D H P A V A R M R V D S D N A Y I G
3175 GTCACCTACAAAAACGAGGAAGACAAGCTGAAGGACTGGGAGGGTGGTCTGGATGAGCAGAGACTGAGC
V T Y K N E E D K L K D W E G G L D E Q R L S
3244 GCTGACAGTGGCTACATCATTCCCTGCCTGACATTGACCCCTGTCCCTGAGGAGGGAGGACCTGGCAAG
A D S G Y I I P L P D I D P V P E E E D L G K
3313 AGGAACAGACACAGCTCGCAGACCTCTGAAGAGAGTGCCATTGAGACGGGTTCCAGCAGTCCACCTC
R N R H S S Q T S E E S A I E T G S S S S T F
3382 ATCAAGAGAGAGGACGAGACATTGAAGACATCGACATGATGGACGACATGGCATAGACTCTCAGAC
I K R E D E T I E D I D M M D D I G I D S S D
3451 CTGGTGGAAAGACAGCTTCCCTGTAACTGGCGGATTGAGGGGTTCTTCACTTCTGGGCCACCTCTGG
L V E D S F L
1089
3520 ATCCCGTTCAGAAAACCACTTATTGCAATGCGGAGGTTGAGAGGGAGCTGGTTGATGTTAAAGAG
3589 AAGTTCCCAGCCAAGGGCTCGGGGAGCGTTCTAAATATGAATGAATGGATATTGAAATGAACCTT
3658 GTCACTGTTGCCTCTGCAATGCCTCAGTAGCATCTCAGTGGTGTGAGAGTTGGAGATAGATGGATA
3727 AGGGATAATAGGCCACAGAAGGTGAACTTGTGCTCAAGGACATTGGTGGAGAGTCCAACAGACACAA
3796 TTTATACTGCGACAGAACCTCAGCATTGTAATTATGAAATAACTCTAACCAAGGCTGTGTTAGATTG
3865 TATTAACATCTTCTTGGACTCTGAAGAGACCACTCAATCCATCCTGTACTTCCCTCTGAAACCTG
3934 ATGTAGCTGCTGTGAACTTTAAAGAAGTGCATGAAAACCATTGAAACCTTAAAGGTACTGGT
4003 ACTATAGCATTGCTATCTTTAGTGTAAAGAGATAAAGAATAAG

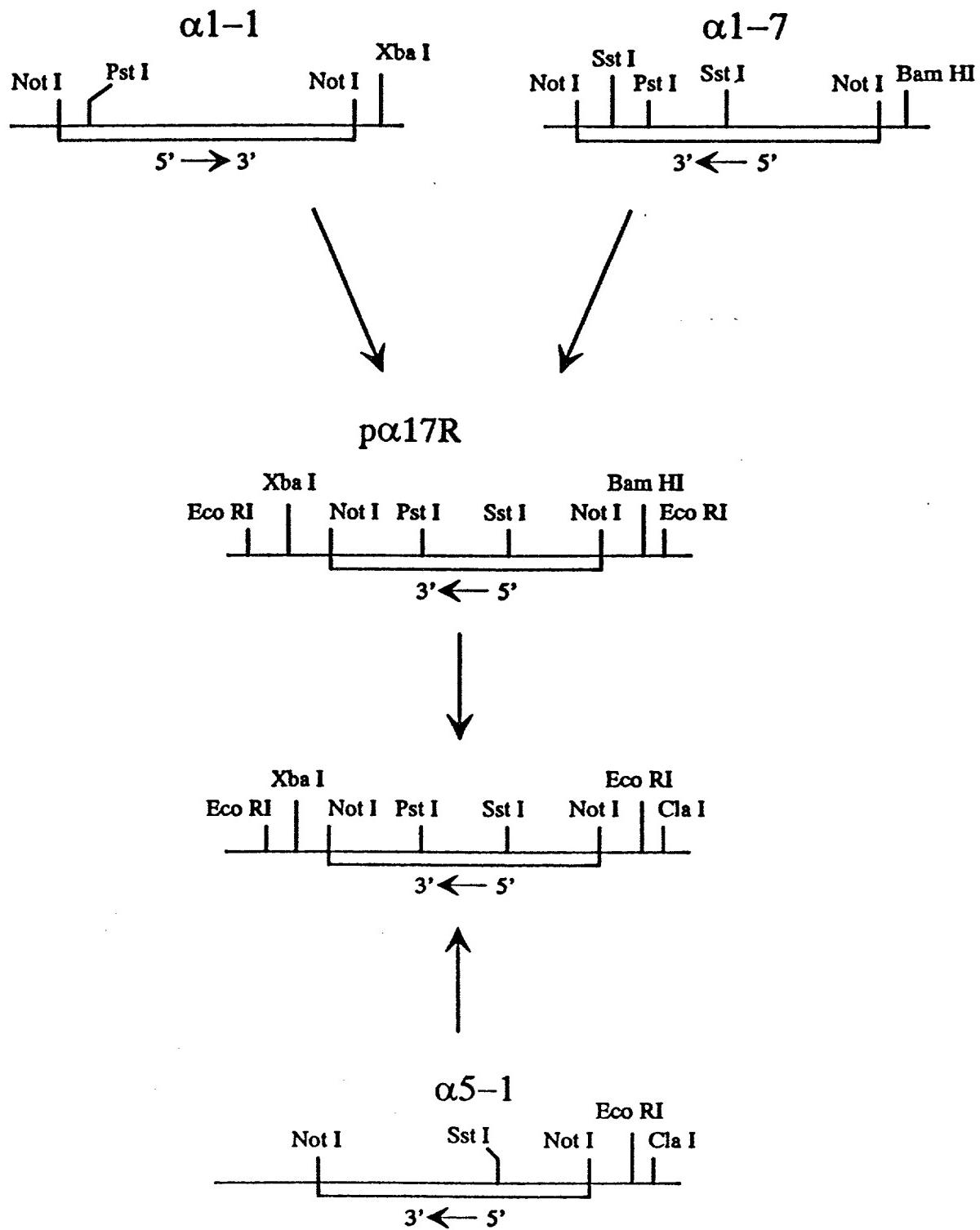


Figure 12

DescriptionMETHODS OF PRODUCING SECRETED RECEPTOR ANALOGS AND
BIOLOGICALLY ACTIVE DIMERIZED POLYPEPTIDE FUSIONS

5

Cross Reference to Related Application

This application is a continuation-in-part of U.S. Application Serial No. 07/347,291, filed May 2, 1989, which is a continuation-in-part application of U.S. 10 Application Serial No. 146,877, filed January 22, 1988, now abandoned.

Technical Field

The present invention is generally directed 15 toward the expression of proteins, and more specifically, toward the expression of growth factor receptor analogs and biologically active dimerized polypeptide fusions.

Background of the Invention

In higher eucaryotic cells, the interaction 20 between receptors and ligands (e.g., hormones) is of central importance in the transmission of and response to a variety of extracellular signals. It is generally accepted that hormones and growth factors elicit their 25 biological functions by binding to specific recognition sites (receptors) in the plasma membranes of their target cells. Upon ligand binding, a receptor undergoes a conformational change, triggering secondary cellular responses that result in the activation or inhibition of 30 intracellular processes. The stimulation or blockade of such an interaction by pharmacological means has important therapeutic implications for a wide variety of illnesses.

Ligands fall into two classes: those that have 35 stimulatory activity, termed agonists; and those that block the effects elicited by the original ligands, termed antagonists. The discovery of agonists that differ in structure and composition from the original ligand may be

medically useful. In particular, agonists that are smaller than the original ligand may be especially useful. The bioavailability of these smaller agonists may be greater than that of the original ligand. This may be of 5 particular importance for topical applications and for instances when diffusion of the agonist to its target sites is inhibited by poor circulation. Agonists may also have slightly different spectra of biological activity and/or different potencies, allowing them to be used in 10 very specific situations. Agonists that are smaller and chemically simpler than the native ligand may be produced in greater quantity and at lower cost. The identification of antagonists which specifically block, for example, growth factor receptors has important pharmaceutical 15 applications. Antagonists that block receptors against the action of endogenous, native ligand may be used as therapeutic agents for conditions including atherosclerosis, autocrine tumors, fibroplasia and keloid formation.

20 The discovery of new ligands that may be used in pharmaceutical applications has centered around designing compounds by chemical modification, complete synthesis, and screening potential ligands by complex and costly screening procedures. The process of designing a new 25 ligand usually begins with the alteration of the structure of the original effector molecule. If the original effector molecule is known to be chemically simple, for example, a catecholamine or prostaglandin, the task is relatively straightforward. However, if the ligand is 30 structurally complex, for example, a peptide hormone or a growth factor, finding a molecule which is functionally equivalent to the original ligand becomes extremely difficult.

Currently, potential ligands are screened using 35 radioligand binding methods (Lefkowitz et al., Biochem. Biophys. Res. Comm. 60: 703-709, 1974; Aurbach et al., Science 186: 1223-1225, 1974; Atlas et al., Proc. Natl.

Acad. Sci. USA 71: 4246-4248, 1974). Potential ligands can be directly assayed by binding the radiolabeled compounds to responsive cells, to the membrane fractions of disrupted cells, or to solubilized receptors.

- 5 Alternatively, potential ligands may be screened by their ability to compete with a known labeled ligand for cell surface receptors.

The success of these procedures depends on the availability of reproducibly high quality preparations of membrane fractions or receptor molecules, as well as the isolation of responsive cell lines. The preparation of membrane fractions and soluble receptor molecules involves extensive manipulations and complex purification steps. The isolation of membrane fractions requires gentle manipulation of the preparation, a procedure which does not lend itself to commercial production. It is very difficult to maintain high biological activity and biochemical purity of receptors when they are purified by classical protein chemistry methods. Receptors, being integral membrane proteins, require cumbersome purification procedures, which include the use of detergents and other solvents that interfere with their biological activity. The use of these membrane preparations in ligand binding assays typically results in low reproducibility due to the variability of the membrane preparations.

As noted above, ligand binding assays require the isolation of responsive cell lines. Often, only a limited subset of cells is responsive to a particular agent, and such cells may be responsive only under certain conditions. In addition, these cells may be difficult to grow in culture or may possess a low number of receptors. Currently available cell types responsive to platelet-derived growth factor (PDGF), for example, contain only a low number (up to 4×10^5 ; see Bowen-Pope and Ross, J. Biol. Chem. 257: 5161-5171, 1982) of receptors per cell,

thus requiring large numbers of cells to assay PDGF analogs or antagonists.

Presently, only a few naturally-occurring secreted receptors, for example, the interleukin-2 receptor (IL-2-R) have been identified. Rubin et al. (J. Immun. 135: 3172-3177, 1985) have reported the release of large quantities of IL-2-R into the culture medium of activated T-cell lines. Bailon et al. (Bio/Technology 5: 1195-1198, 1987) have reported the use of a matrix-bound interleukin-2 receptor to purify recombinant interleukin-2.

Three other receptors have been secreted from mammalian cells. The insulin receptor (Ellis et al., J. Cell Biol. 150: 14a, 1987), the HIV-1 envelope glycoprotein cellular receptor CD4 (Smith et al., Science 238: 1704-1707, 1987), the murine IL-7 receptor (Cell 60: 941-951, 1990) and the epidermal growth factor (EGF) receptor (Livneh et al., J. Biol. Chem. 261: 12490-12497, 1986) have been secreted from mammalian cells using truncated cDNAs that encode portions of the extracellular domains.

Naturally-occurring, secreted receptors have not been widely identified, and there have been only a limited number of reports of secreted recombinant receptors. Secreted receptors may be used in a variety of assays, which include assays to determine the presence of ligand in biological fluids and assays to screen for potential agonists and antagonists. Current methods for ligand screening and ligand/receptor binding assays have been limited to those using preparations of whole cells or cell membranes for as a source for receptor molecules. The low reproducibility and high cost of producing such preparations does not lend itself to commercial production. There is therefore a need in the art for a method of producing secreted receptors. There is a further need in the art for an assay system that permits high volume screening of compounds that may act on higher eucaryotic cells via specific surface receptors. This

assay system should be rapid, inexpensive and adaptable to high volume screening. The present invention discloses such a method and assay system, and further provides other related advantages.

5

Disclosure of Invention

Briefly stated, the present invention discloses methods for producing secreted receptor analogs, including receptor analogs and secreted platelet-derived growth factor receptor (PDGF-R) analogs. In addition, the present invention discloses methods for producing secreted biologically active dimerized polypeptide fusions.

Within one aspect of the invention a method for producing a secreted PDGF-R analog is disclosed, comprising (a) introducing into a eukaryotic host cell a DNA construct comprising a transcriptional promoter operatively linked to a secretory signal sequence followed downstream of and in proper reading frame with a DNA sequence encoding at least a portion of the ligand-binding domain of a PDGF-R, the portion including a ligand-binding domain; (b) growing the host cell in an appropriate growth medium under physiological conditions to allow the secretion of a PDGF-R analog encoded by said DNA sequence; and (c) isolating the PDGF-R analog from the host cell.

Within one embodiment of the present invention, a PDGF-R analog comprising the amino acid sequence of Figure 1 (Sequence ID Numbers 1 and 2) from isoleucine, number 29, to methionine, number 441, is secreted. Within another embodiment, a PDGF-R analog comprising the amino acid sequence of Figure 1 (Sequence ID Numbers 1 and 2) from isoleucine, number 29 to lysine, number 531 is secreted. Within yet another embodiment of the invention, a PDGF-R analog comprising the amino acid sequence of Figure 11 (Sequence ID Numbers 35 and 36) from glutamine, number 24 to glutamic acid, number 524 is secreted.

Yet another aspect of the present invention discloses a method for producing a secreted, biologically

active dimerized polypeptide fusion. The method generally comprises a) introducing into a eukaryotic host cell a DNA construct comprising a transcriptional promoter operatively linked to a secretory signal sequence followed 5 downstream by and in proper reading frame with a DNA sequence encoding a non-immunoglobulin polypeptide requiring dimerization for biological activity joined to a dimerizing protein; (b) growing the host cell in an appropriate growth medium under physiological conditions 10 to allow the secretion of a dimerized polypeptide fusion encoded by said DNA sequence; and (c) isolating the biologically active dimerized polypeptide fusion from the host cell.

Within one embodiment, the dimerizing protein is 15 yeast invertase. Within another embodiment, the dimerizing protein is at least a portion of an immunoglobulin light chain. Within another embodiment, the dimerizing protein is at least a portion of an immunoglobulin heavy chain.

In another aspect of the invention, a method is disclosed for producing a secreted, biologically active dimerized polypeptide fusion, comprising (a) introducing into a eukaryotic host cell a first DNA construct comprising a transcriptional promoter operatively linked 20 to a first secretory signal sequence followed downstream by and in proper reading frame with a first DNA sequence encoding a non-immunoglobulin polypeptide requiring dimerization for biological activity joined to an immunoglobulin light chain constant region; (b) introducing into the host cell a second DNA construct comprising a transcriptional promoter operatively linked 25 to a second secretory signal sequence followed downstream by and in proper reading frame with a second DNA sequence encoding an immunoglobulin heavy chain constant region 30 domain selected from the group consisting of C_H1, C_H2, C_H3, and C_H4; (c) growing the host cell in an appropriate growth medium under physiological conditions to allow the 35

secretion of a dimerized polypeptide fusion encoded by said first and second DNA sequences; and (d) isolating the dimerized polypeptide fusion from the host cell. In one embodiment, the second DNA sequence further encodes an immunoglobulin heavy chain hinge region wherein the hinge region is joined to the heavy chain constant region domain. In a preferred embodiment, the second DNA sequence further encodes an immunoglobulin variable region joined upstream of and in proper reading frame with the immunoglobulin heavy chain constant region.

In another aspect of the invention, a method is disclosed for producing a secreted, biologically active dimerized polypeptide fusion, comprising (a) introducing into a eukaryotic host cell a first DNA construct comprising a transcriptional promoter operatively linked to a first secretory signal sequence followed downstream by and in proper reading frame with a first DNA sequence encoding a non-immunoglobulin polypeptide requiring dimerization for biological activity joined to an immunoglobulin heavy chain constant region domain selected from the group consisting of C_H1, C_H2, C_H3, and C_H4; (b) introducing into the host cell a second DNA construct comprising a transcriptional promoter operatively linked to a second secretory signal sequence followed downstream by and in proper reading frame with a second DNA sequence encoding an immunoglobulin light chain constant region; (c) growing the host cell in an appropriate growth medium under physiological conditions to allow the secretion of a dimerized polypeptide fusion encoded by said first and second DNA sequences; and (d) isolating the dimerized polypeptide fusion from the host cell. In one embodiment, the first DNA sequence further encodes an immunoglobulin heavy chain hinge region wherein the hinge region is joined to the heavy chain constant region domain. In a preferred embodiment, the second DNA sequence further encodes an immunoglobulin variable region joined upstream

of and in proper reading frame with the immunoglobulin light chain constant region.

In another aspect of the invention, a method is disclosed for producing a secreted, biologically active 5 dimerized polypeptide fusion, comprising (a) introducing into a eukaryotic host cell a DNA construct comprising a transcriptional promoter operatively linked to a secretory signal sequence followed downstream by and in proper reading frame with a DNA sequence encoding a non- 10 immunoglobulin polypeptide requiring dimerization for biological activity joined to an immunoglobulin heavy chain constant region domain selected from the group consisting of C_H1, C_H2, C_H3, and C_H4; (b) growing the host cell in an appropriate growth medium under physiological 15 conditions to allow the secretion of a dimerized polypeptide fusion encoded by said first and second DNA sequences; and (c) isolating the biologically active dimerized polypeptide fusion from the host cell. In one embodiment, the DNA sequence further encodes an 20 immunoglobulin heavy chain hinge region wherein the hinge region is joined to the heavy chain constant region domain.

In another aspect of the invention, a method is disclosed for producing a secreted, biologically active 25 dimerized polypeptide fusion, comprising (a) introducing into a eukaryotic host cell a first DNA construct comprising a transcriptional promoter operatively linked to a first secretory signal sequence followed downstream by and in proper reading frame with a first DNA sequence 30 encoding a first polypeptide chain of a non-immunoglobulin polypeptide dimer requiring dimerization for biological activity joined to an immunoglobulin heavy chain constant region domain, selected from the group consisting of C_H1, C_H2, C_H3, and C_H4; (b) introducing into the host cell a 35 second DNA construct comprising a transcriptional promoter operatively linked to a second secretory signal sequence followed downstream by and in proper reading frame with a

second DNA sequence encoding a second polypeptide chain of the non-immunoglobulin polypeptide dimer joined to an immunoglobulin light chain constant region domain; (c) growing the host cell in an appropriate growth medium
5 under physiological conditions to allow the secretion of a dimerized polypeptide fusion encoded by said first and second DNA sequences wherein said dimerized polypeptide fusion exhibits biological activity characteristic of said non-immunoglobulin polypeptide dimer; and (d) isolating
10 the dimerized polypeptide fusion from the host cell. In one embodiment the first DNA sequence further encodes an immunoglobulin heavy chain hinge region domain wherein the hinge region is joined to the immunoglobulin heavy chain constant region domain.

15 Within one embodiment of the present invention, a biologically active dimerized polypeptide fusion comprising the amino acid sequence of Figure 1 (Sequence ID Numbers 1 and 2) from isoleucine, number 29, to methionine, number 441, is secreted. Within another
20 embodiment, a biologically active dimerized polypeptide fusion comprising the amino acid sequence of Figure 1 (Sequence ID Numbers 1 and 2) from isoleucine, number 29 to lysine, number 531 is secreted. Within another embodiment of the invention, a biologically active
25 dimerized polypeptide fusion comprising the amino acid sequence of Figure 11 (Sequence ID Numbers 35 and 36) from glutamine, number 24 to glutamic acid, number 524 is secreted. Within yet another embodiment of the invention, a biologically active dimerized polypeptide fusion
30 comprising the amino acid sequence of Figure 1 (Sequence ID Numbers 1 and 2) from isoleucine, number 29 to lysine, number 531 dimerized to the amino acid sequence of Figure 11 (Sequence ID Numbers 35 and 36) from glutamine, number 24 to glutamic acid, number 524 is secreted.

35 In yet another aspect of the invention, a method is disclosed for producing a secreted receptor analog, comprising (a) introducing into a eukaryotic host cell a

DNA construct comprising a transcriptional promoter operatively linked to at least one secretory signal sequence followed downstream by and in proper reading frame with a DNA sequence encoding a ligand-binding domain
5 of a receptor requiring dimerization for biological activity joined to a dimerizing protein; (b) growing the host cell in an appropriate growth medium under physiological conditions to allow the secretion of a receptor analog encoded by said DNA sequence; and (c)
10 isolating the receptor analog from the host cell.

In yet another aspect of the invention, a method is disclosed for producing a secreted receptor analog, comprising (a) introducing into a eukaryotic host cell a first DNA construct comprising a transcriptional promoter operatively linked to a first secretory signal sequence followed downstream by and in proper reading frame with a first DNA sequence encoding a ligand-binding domain of a receptor requiring dimerization for biological activity joined to an immunoglobulin light chain constant region;
15 (b) introducing into the host cell a second DNA construct comprising a transcriptional promoter operatively linked to a second secretory signal sequence followed downstream by and in proper reading frame with a second DNA sequence encoding an immunoglobulin heavy chain constant region
20 domain, selected from the group consisting of C_H1, C_H2, C_H3, and C_H4; (c) growing the host cell in an appropriate growth medium under physiological conditions to allow the secretion of a receptor analog encoded by said first and second DNA sequences; and (d) isolating the receptor
25 analog from the host cell. In one embodiment, the second DNA sequence further encodes an immunoglobulin heavy chain hinge region wherein the hinge region is joined to the heavy chain constant region domain. In a preferred embodiment, the second DNA sequence further encodes an
30 immunoglobulin variable region joined upstream of and in proper reading frame with the immunoglobulin heavy chain constant region.
35

In another aspect of the invention, a method is disclosed for producing a secreted receptor analog, comprising (a) introducing into a eukaryotic host cell a DNA construct comprising a transcriptional promoter operatively linked to a secretory signal sequence followed downstream by and in proper reading frame with a DNA sequence encoding a ligand-binding domain of a receptor requiring dimerization for biological activity joined to an immunoglobulin heavy chain constant region domain, selected from the group C_H1, C_H2, C_H3, and C_H4; (b) growing the host cell in an appropriate growth medium under physiological conditions to allow the secretion of the receptor analog; and (c) isolating the receptor analog from the host cell. In one embodiment, the DNA sequence further encodes an immunoglobulin heavy chain hinge region wherein the hinge region is joined to the heavy chain constant region domain.

In another aspect of the invention, a method is disclosed for producing a secreted receptor analog, comprising (a) introducing into a eukaryotic host cell a first DNA construct comprising a transcriptional promoter operatively linked to a first secretory signal sequence followed downstream of and in proper reading frame with a first DNA sequence encoding a ligand-binding domain of a receptor requiring dimerization for biological activity joined to an immunoglobulin heavy chain constant region domain, selected from the group C_H1, C_H2, C_H3, and C_H4; (b) introducing into the host cell a second DNA construct comprising a transcriptional promoter operatively linked to a second secretory signal sequence followed downstream by and in proper reading frame with a second DNA sequence encoding an immunoglobulin light chain constant region; (c) growing the host cell in an appropriate growth medium under physiological conditions to allow the secretion of a receptor analog encoded by said first and second DNA sequences; and (d) isolating the receptor analog from the host cell. In one embodiment, the first DNA sequence

further encodes an immunoglobulin heavy chain hinge region wherein the hinge region is joined to the heavy chain constant region domain. In a preferred embodiment, the second DNA sequence further encodes an immunoglobulin
5 variable region joined upstream of and in proper reading frame with the immunoglobulin light chain constant region.

In another aspect of the invention, a method is disclosed for producing a secreted receptor analog, comprising (a) introducing into a eukaryotic host cell a
10 first DNA construct comprising a transcriptional promoter operatively linked to a first secretory signal sequence followed downstream in proper reading frame by a first DNA sequence encoding a first polypeptide chain of a ligand-binding domain of a receptor requiring dimerization for
15 biological activity joined to an immunoglobulin heavy chain constant region domain, selected from the group C_{H1} , C_{H2} , C_{H3} , and C_{H4} ; (b) introducing into the host cell a second DNA construct comprising a transcriptional promoter operatively linked to a second secretory signal sequence
20 followed downstream by and in proper reading frame with a second DNA sequence encoding a second polypeptide chain of the ligand-binding domain of said receptor joined to an immunoglobulin light chain constant region domain; (c) growing the host cell in an appropriate growth medium
25 under physiological conditions to allow the secretion of a receptor analog encoded by said first and second DNA sequences; and (d) isolating the receptor analog from the host cell. In one embodiment the first DNA sequence further encodes an immunoglobulin heavy chain hinge region
30 domain wherein the hinge region is joined to the immunoglobulin heavy chain constant region domain.

Host cells for use in the present invention include cultured mammalian cells and fungal cells. In a preferred embodiment strains of the yeast Saccharomyces
35 cerevisiae are used as host cells. Within another preferred embodiment cultured rodent myeloma cells are used as host cells.

Within one embodiment of the present invention, a receptor analog is a PDGF-R analog comprising the amino acid sequence of Figure 1 (Sequence ID Numbers 1 and 2) from isoleucine, number 29, to methionine, number 441.

5 Within another embodiment a PDGF-R analog comprises the amino acid sequence of Figure 1 (Sequence ID Numbers 1 and 2) from isoleucine, number 29, to lysine, number 531. Within another embodiment of the invention, a PDGF-R analog comprises the amino acid sequence of Figure 11
10 (Sequence ID Numbers 35 and 36) from glutamine, number 24 to glutamic acid, number 524 is secreted. Within yet another embodiment of the invention, a PDGF-R analog comprises the amino acid sequence of Figure 1 (Sequence ID Numbers 1 and 2) from isoleucine, number 29 to lysine,
15 number 531 and the amino acid sequence of Figure 11 (Sequence ID Numbers 35 and 36) from glutamine, number 24 to glutamic acid, number 524 is secreted.

PDGF-R analogs produced by the above-disclosed methods may be used, for instance, within a method for
20 determining the presence of human PDGF or an isoform thereof in a biological sample.

A method for determining the presence of human PDGF or an isoform thereof in a biological sample is disclosed and comprises (a) incubating a polypeptide
25 comprising a PDGF receptor analog fused to a dimerizing protein with a biological sample suspected of containing PDGF or an isoform thereof under conditions that allow the formation of receptor/ligand complexes; and (b) detecting the presence of receptor/ligand complexes, and therefrom
30 determining the presence of PDGF or an isoform thereof. Suitable biological samples in this regard include blood, urine, plasma, serum, platelet and other cell lysates, platelet releasates, cell suspensions, cell-conditioned culture media, and chemically or physically separated
35 portions thereof.

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings.

5 Brief Description of the Drawings

Figure 1 (Sequence ID Numbers 1 and 2) illustrates the nucleotide sequence of a representative PDGF β -receptor cDNA and the derived amino acid sequence of the primary translation product and corresponds to Sequence ID Number 1). Numbers above the lines refer to the nucleotide sequence; numbers below the lines refer to the amino acid sequence.

Figure 2 illustrates the construction of pBTL10, pBTL11 and pBTL12.

15 Figure 3 illustrates the construction of pCBS22.

Figure 4 illustrates the construction of pBTL13 and pBTL14.

Figure 5 illustrates the construction of pBTL15.

20 Figure 6 illustrates the construction of pBTL22 and pBTL26.

Figure 7 illustrates the construction of pSDL114. Symbols used are S.S., signal sequence, C_k, immunoglobulin light chain constant region sequence; μ prom, μ promoter, μ enh; μ enhancer.

25 Figure 8 illustrates the construction of pSDLB113. Symbols used are S.S., signal sequence; C_{H1}, C_{H2}, C_{H3}, immunoglobulin heavy chain constant region domain sequences; H, immunoglobulin heavy chain hinge region sequence; M, immunoglobulin membrane anchor sequences; C_{γ1M}, immunoglobulin heavy chain constant region and membrane anchor sequences.

30 Figure 9 illustrates the constructions pBTL115, pBTL114, pφ5V_HHuC_{γ1M}-neo, pLCφ5V_KHuC_K-neo. Symbols used are set forth in Figures 7 and 8, and also include L_H, mouse immunoglobulin heavy chain signal sequence; V_H, mouse immunoglobulin heavy chain variable region sequence; E, mouse immunoglobulin heavy chain enhancer sequence; L_K,

mouse immunoglobulin light chain signal sequence; ϕ 5V_K, mouse immunoglobulin light chain variable region sequence; Neo^R, E. coli neomycin resistance gene.

Figure 10 illustrates the constructions Zem229R,
5 p ϕ 5V_HFab-neo and pWKI. Symbols used are set forth in Figure 9.

Figure 11 illustrates the sequence of a representative PDGF α -receptor cDNA and the deduced amino acid sequence (using standard one-letter codes) encoded by
10 the cDNA and corresponds to Sequence ID Numbers 35 and 36. Numbers at the ends of the lines refer to nucleotide positions. Numbers below the sequence refer to amino acid positions.

Figure 12 illustrates the assembly of a cDNA
15 molecule encoding a PDGF α -receptor. Complementary DNA sequences are shown as lines. Only those portions of the vectors adjacent to the cDNA inserts are shown.

Detailed Description of the Invention

Prior to setting forth the invention, it may be helpful to an understanding thereof to set forth definitions of certain terms to be used hereinafter.

DNA Construct: A DNA molecule, or a clone of such a molecule, either single- or double-stranded that
25 has been modified through human intervention to contain segments of DNA combined and juxtaposed in a manner that as a whole would not otherwise exist in nature.

DNA constructs contain the information necessary to direct the expression and/or secretion of DNA sequences
30 encoding polypeptides of interest. DNA constructs will generally include promoters, enhancers and transcription terminators. DNA constructs containing the information necessary to direct the secretion of a polypeptide will also contain at least one secretory signal sequence.

Secretory Signal Sequence: A DNA sequence encoding a secretory peptide. A secretory peptide is an amino acid sequence that acts to direct the secretion of a

mature polypeptide or protein from a cell. Secretory peptides are characterized by a core of hydrophobic amino acids and are typically (but not exclusively) found at the amino termini of newly synthesized proteins. Very often 5 the secretory peptide is cleaved from the mature protein during secretion. Such secretory peptides contain processing sites that allow cleavage of the signal peptides from the mature proteins as it passes through the secretory pathway. Processing sites may be encoded within 10 the signal peptide or may be added to the signal peptide by, for example, in vitro mutagenesis. Certain secretory peptides may be used in concert to direct the secretion of polypeptides and proteins. One such secretory peptide that may be used in combination with other secretory 15 peptides is the third domain of the yeast Barrier protein.

Receptor Analog: A non-immunoglobulin polypeptide comprising a portion of a receptor which is capable of binding ligand and/or are recognized by anti-receptor antibodies. The amino acid sequence of the 20 receptor analog may contain additions, substitutions or deletions as compared to the native receptor sequence. A receptor analog may be, for example, the ligand-binding domain of a receptor joined to another protein. Platelet-derived growth factor receptor (PDGF-R) analogs may, for 25 example, comprise a portion of a PDGF receptor capable of binding anti-PDGF receptor antibodies, PDGF, PDGF isoforms, PDGF analogs, or PDGF antagonists.

Dimerizing Protein: A polypeptide chain having affinity for a second polypeptide chain, such that the two 30 chains associate under physiological conditions to form a dimer. The second polypeptide chain may be the same or a different chain.

Biological activity: A function or set of activities performed by a molecule in a biological context 35 (i.e., in an organism or an in vitro facsimile thereof). Biological activities may include the induction of extracellular matrix secretion from responsive cell lines,

the induction of hormone secretion, the induction of chemotaxis, the induction of mitogenesis, the induction of differentiation, or the inhibition of cell division of responsive cells. A recombinant protein or peptide is 5 considered to be biologically active if it exhibits one or more biological activities of its native counterpart.

Ligand: A molecule capable of being bound by the ligand-bindind domain of a receptor or by a receptor analog. The molecule may be chemically synthesized or may 10 occur in nature. Ligands may be grouped into agonists and antagonists. Agonists are those molecules whose binding to a receptor induces the response pathway within a cell. Antagonists are those molecules whose binding to a receptor blocks the response pathway within a cell.

15 Joined: Two or more DNA coding sequences are said to be joined when, as a result of in-frame fusions between the DNA coding sequences or as a result of the removal of intervening sequences by normal cellular processing, the DNA coding sequences are translated into a 20 polypeptide fusion.

As noted above, the present invention provides methods for producing biologically active dimerized polypeptide fusions and secreted receptor analogs, which 25 include, for example, PDGF receptor analogs. Secreted receptor analogs may be used to screen for new compounds that act as agonists or antagonists when interacting with cells containing membrane-bound receptors. In addition, the methods of the present invention provide dimerized 30 non-immunoglobulin polypeptide fusions of therapeutic value that are biologically active only as dimers. Moreover, the present invention provides methods of producing polypeptide dimers that are biologically active only as non-covalently associated dimers. Secreted, 35 biologically active dimers that may be produced using the present invention include nerve growth factor, colony

stimulating factor-1, factor XIII, and transforming growth factor β .

As used herein, the ligand-binding domain of a receptor is that portion of the receptor that is involved with binding the natural ligand. While not wishing to be bound by theory, the binding of a natural ligand to a receptor is believed to induce a conformational change which elicits a response to the change within the response pathway of the cell. For membrane-bound receptors, the ligand-binding domain is generally believed to comprise the extracellular doamin for the receptor. The structure of receptors may be predicted from the primary translation products using the hydrophobicity plot function of, for example, P/C Gene or Intelligenetics Suite (Intelligenetics, Mt. View, CA) or may be predicted according to the methods described, for example, by Kyte and Doolittle, J. Mol. Biol. 157:105-132, 1982). The ligand-binding domain of the PDGF β -receptor, for example, has been predicted to include amino acids 29-531 of the published sequence (Gronwald et al., ibid.). The ligand-binding domain of the PDGF α -receptor has been predicted to include amino acids 25-500 of the published α -receptor sequence (Matsui et al., ibid.). As used herein, the ligand-binding domain of the PDGF β -receptor includes amino acids 29-441 of the sequence of Figure 1 (Sequence ID Number 1) and C-terminal extensions up to and including amino acid 531. The ligand-binding domain of the PDGF α -receptor is understood to include amino acids 24-524 of Figure 11 (Sequence ID Numbers 35 and 36).

Receptor analogs that may be used in the present invention include the ligand-binding domains of the epidermal growth factor receptor (EGF-R) and the insulin receptor. As used herein, a ligand-binding domain is that portion of the receptor that is involved in binding ligand and is generally a portion or essentially all of the extracellular domain that extends from the plasma membrane into the extracellular space. The ligand-binding domain

of the EGF-R, for example, resides in the extracellular domain. EGF-R dimers have been found to exhibit higher ligand-binding affinity than EGF-R monomers (Bonisch-Schnetzler and Pilch, Proc. Natl. Acad. Sci. USA 84:7832-5 7836, 1987). The insulin receptor (Ullrich et al., Nature 313:756-761, 1985) requires dimerization for biological activity.

Another example of a receptor that may be secreted from a host cell is a platelet-derived growth factor receptor (PDGF-R). Two classes of PDGF-Rs, which recognized different isoforms of PDGF, have been identified. (PDGF is a disulfide-bonded, two-chain molecule, which is made up of an A chain and a B chain. These chains may be combined as AB heterodimers, AA homodimers or BB homodimers. These dimeric molecules are referred to herein as "isoforms".) The β -receptor (PDGF β -R), which recognizes only the BB isoform of PDGF (PDGF-BB), has been described (Claesson-Welsh et al., Mol. Cell. Biol. 8:3476-3486, 1988; Gronwald et al., Proc. Natl. Acad. Sci. USA 85:3435-3439, 1988). The α -receptor (PDGF α -R), which recognizes all three PDGF isoforms (PDGF-AA, PDGF-AB and PDGF-BB), has been described by Matsui et al. (Science 243:800-804, 1989) and Kelly and Murray (pending commonly assigned U.S. Patent Application Serial No. 25 07/355,018, which is incorporated herein by reference). The primary translation products of these receptors indicate that each includes an extracellular domain implicated in the ligand-binding process, a transmembrane domain, and a cytoplasmic domain containing a tyrosine kinase activity.

The present invention provides a standardized assay system, not previously available in the art, for determining the presence of PDGF, PDGF isoforms, PDGF agonists or PDGF antagonists using a secreted PDGF receptor analogs. Such an assay system will typically involve combining the secreted PDGF receptor analog with a biological sample under physiological conditions which

permit the formation of receptor-ligand complexes, followed by detecting the presence of the receptor-ligand complexes. The term physiological conditions is meant to include those conditions found within the host organism
5 and include, for example, the conditions of osmolarity, salinity and pH. Detection may be achieved through the use of a label attached to the PDGF receptor analog or through the use of a labeled antibody which is reactive with the receptor analog or the ligand. A wide variety of
10 labels may be utilized, such as radionuclides, fluorophores, enzymes and luminescers. Receptor-ligand complexes may also be detected visually, i.e., in immunoprecipitation assays which do not require the use of a label. This assay system provides secreted PDGF
15 receptor analogs that may be utilized in a variety of screening assays for, for example, screening for analogs of PDGF. The present invention also provides a methods for measuring the level of PDGF and PDGF isoforms in biological fluids.

20 As noted above, the present invention provides methods for producing dimerized polypeptide fusions that require dimerization for biological activity or enhancement of biological activity. Polypeptides requiring dimerization for biological activity include, in
25 addition to certain receptors, nerve growth factor, colony-stimulating factor-1 (CSF-1), transforming growth factor β (TGF- β), PDGF, and factor XIII. Nerve growth factor is a non-covalently linked dimer (Harper et al., J. Biol. Chem. 257: 8541-8548, 1982). CSF-1, which
30 specifically stimulates the proliferation and differentiation of cells of mononuclear phagocytic lineage, is a disulfide-bonded homodimer (Retternmier et al., Mol. Cell. Biol. 7: 2378-2387, 1987). TGF- β is biologically active as a disulfide-bonded dimer (Assoian et al., J. Biol. Chem. 258: 7155-7160, 1983). Factor XIII
35 is a plasma protein that exists as a two chain homodimer in its activated form (Ichinose et al., Biochem. 25: 6900-

6906, 1986). PDGF, as noted above, is a disulfide-bonded, two chain molecule (Murray et al., U.S. Patent 4,766,073).

The present invention provides methods by which receptor analogs, including receptor analogs and PDGF-R analogs, requiring dimerization for activity may be secreted from host cells. The methods described herein are particularly advantageous in that they allow the production of large quantities of purified receptors. The receptors may be used in assays for the screening of potential ligands, in assays for binding studies, as imaging agents, and as agonists and antagonists within therapeutic agents.

A DNA sequence encoding a human PDGF receptor may be isolated as a cDNA using techniques known in the art (see, for example, Okayama and Berg, Mol. Cell. Biol. 2: 161-170, 1982; Mol. Cell. Biol. 3: 280-289, 1983) from a library of human genomic or cDNA sequences. Such libraries may be prepared by standard procedures, such as those disclosed by Gubler and Hoffman (Gene 25: 263-269, 1983). It is preferred that the molecule is a cDNA molecule because cDNA lack introns and are therefore more suited to manipulation and expression in transfected or transformed cells. Sources of mRNA for use in the preparation of a cDNA library include the MG-63 human osteosarcoma cell line (available from ATCC under accession number CRL 1427), diploid human dermal fibroblasts and human embryo fibroblast and brain cells (Matsui et al., *ibid.*). A cDNA encoding a PDGF β -R has been cloned from a diploid human dermal fibroblast cDNA library using oligonucleotide probes complementary to sequences of the mouse PDGF receptor (Gronwald et al., *ibid.*). A PDGF α -R cDNA has been isolated by Matsui et al. (*ibid.*) from human embryo fibroblast and brain cells. Alternatively, a cDNA encoding a PDGF α -R may be isolated from a library prepared from MG-63 human osteosarcoma cells using a cDNA probe containing sequences encoding the transmembrane and cytoplasmic domains of the PDGF β -R.

(described by Kelly and Murray, *ibid.*). Partial cDNA clones (fragments) can be extended by re-screening of the library with the cloned cDNA fragment until the full sequence is obtained. In one embodiment, a ligand-binding domain of a PDGF receptor is encoded by the sequence of Figure 1 (Sequence ID Number 1) from amino acid 29 through amino acid 441. In another embodiment, a ligand-binding domain of a PDGF receptor is encoded by the sequence of Figure 1 (Sequence ID Number 1) from amino acid 29 through 10 amino acid 531. In yet another embodiment, a ligand-binding domain of a PDGF receptor is encoded by the sequence of Figure 11 (Sequence ID Numbers 35 and 36) from amino acid 24 through amino acid 524. One skilled in the art may envision the use of smaller DNA sequence encoding 15 the ligand-binding domain of a PDGF receptor containing at least 400 amino acids of the extracellular domain.

DNA sequences encoding EGF-R (Ullrich et al., *Nature* 304: 418-425, 1984), the insulin receptor (Ullrich et al., *Nature* 313: 756-761, 1985), nerve growth factor 20 (Ullrich et al. *Nature* 303: 821-825, 1983), colony stimulating factor-1 (Rettenmier et al., *ibid.*), transforming growth factor β (Derynck et al., *Nature* 316: 701-705, 1985), PDGF (Murray et al., *ibid.*), and factor XIII (Ichinose et al., *ibid.*) may also be used within the 25 present invention.

To direct polypeptides requiring dimerization for biological activity or receptor analogs into the secretory pathway of the host cell, at least one secretory signal sequence is used in conjunction with the DNA 30 sequence of interest. Preferred secretory signals include the alpha factor signal sequence (pre-pro sequence) (Kurjan and Herkowitz, *Cell* 30: 933-943, 1982; Kurjan et al., U.S. Patent No. 4,546,082; Brake, EP 116,201, 1983), the PHO5 signal sequence (Beck et al., WO 86/00637), the 35 BAR1 secretory signal sequence (MacKay et al., U.S. Patent No. 4,613,572; MacKay, WO 87/002670), immunoglobulin V_H signal sequences (Loh et al., *Cell* 33: 85-93, 1983; Watson

Nuc. Acids. Res. 12: 5145-5164, 1984) and immunoglobulin V_k signal sequences (Watson, ibid.). Particularly preferred signal sequences are the SUC2 signal sequence (Carlson et al., Mol. Cell. Biol. 3: 439-447, 1983) and

- 5 PDGF receptor signal sequences. Alternatively, secretory signal sequences may be synthesized according to the rules established, for example, by von Heinje (Eur. J. Biochem. 133: 17-21, 1983; J. Mol. Biol. 184: 99-105, 1985; Nuc. Acids. Res. 14: 4683-3690, 1986).

10 Secretory signal sequences may be used singly or may be combined. For example, a first secretory signal sequence may be used singly or combined with a sequence encoding the third domain of Barrier (described in co-pending commonly assigned U.S. Patent Application Serial 15 No. 07/104,316, which is incorporated by reference herein in its entirety). The third domain of Barrier may be positioned in proper reading frame 3' of the DNA sequence of interest or 5' to the DNA sequence and in proper reading frame with both the secretory signal sequence and 20 the DNA sequence of interest.

In one embodiment of the present invention, a sequence encoding a dimerizing protein is joined to a sequence encoding a polypeptide chain of a polypeptide dimer or a receptor analog, and this fused sequence is 25 joined in proper reading frame to a secretory signal sequence. As shown herein, the present invention utilizes such an arrangement to drive the association of the polypeptide or receptor analog to form a biologically active molecule upon secretion. Suitable dimerizing 30 proteins include the S. cerevisiae repressible acid phosphatase (Mizunaga et al., J. Biochem. (Tokyo) 103: 321-326, 1988), the S. cerevisiae type 1 killer preprotoxin (Sturley et al., EMBO J. 5: 3381-3390, 1986), the S. calsbergensis alpha galactosidase melibiase 35 (Sumner-Smith et al., Gene 36: 333-340, 1985), the S. cerevisiae invertase (Carlson et al., Mol. Cell. Biol. 3: 439-447, 1983), the Neurospora crassa ornithine

decarboxylase (Digangi et al., J. Biol. Chem. **262**: 7889-7893, 1987), immunoglobulin heavy chain hinge regions (Takahashi et al., Cell **29**: 671-679, 1982), and other dimerizing immunoglobulin sequences. In a preferred embodiment, S. cerevisiae invertase is used to drive the association of polypeptides into dimers. Portions of dimerizing proteins, such as those mentioned above, may be used as dimerizing proteins where those portions are capable of associating as a dimer in a covalent or noncovalent manner. Such portions may be determined by, for example, altering a sequence encoding a dimerizing protein through in vitro mutagenesis to delete portions of the coding sequence. These deletion mutants may be expressed in the appropriate host to determine which portions retain the capability of associating as dimers. Portions of immunoglobulin gene sequences may be used to drive the association of non-immunoglobulin polypeptides. These portions correspond to discrete domains of immunoglobulins. Immunoglobulins comprise variable and constant regions, which in turn comprise discrete domains that show similarity in their three-dimensional conformations. These discrete domains correspond to immunoglobulin heavy chain constant region domain exons, immunoglobulin heavy chain variable region domain exons, immunoglobulin light chain variable region domain exons and immunoglobulin light chain constant region domain exons in immunoglobulin genes (Hood et al., in Immunology, The Benjamin/Cummings Publishing Company, Inc., Menlo Park, CA; Honjo et al., Cell **18**: 559-568, 1979; Takahashi et al., Cell **29**: 671-679, 1982; and Honjo, Ann. Rev. Immun. **1**:499-528, 1983)). Particularly preferred portions of immunoglobulin heavy chains include Fab and Fab' fragments. (An Fab fragment is a portion of an immunoglobulin heavy chain that includes a heavy chain variable region domain and a heavy chain constant region domain. An Fab' fragment is a portion of an immunoglobulin heavy chain that includes a heavy chain

variable region domain, a heavy chain constant region domain and a heavy chain hinge region domain.)

It is preferred to use an immunoglobulin light chain constant region in association with at least one immunoglobulin heavy chain constant region domain. In another embodiment, an immunoglobulin light chain constant region is associated with at least one immunoglobulin heavy chain constant region domain joined to an immunoglobulin hinge region. In one set of embodiments, an immunoglobulin light chain constant region joined in frame with a polypeptide chain of a non-immunoglobulin polypeptide dimer or receptor analog and is associated with at least one heavy chain constant region. In a preferred set of embodiments a variable region is joined upstream of and in proper reading frame with at least one immunoglobulin heavy chain constant region. In another set of embodiments, an immunoglobulin heavy chain is joined in frame with a polypeptide chain of a non-immunoglobulin polypeptide dimer or receptor analog and is associated with an immunoglobulin light chain constant region. In yet another set of embodiments, a polypeptide chain of a non-immunoglobulin polypeptide dimer or receptor analog joined is to at least one immunoglobulin heavy chain constant region which is joined to an immunoglobulin hinge region and is associated with an immunoglobulin light chain constant region. In a preferred set of embodiments an immunoglobulin variable region is joined upstream of and in proper reading frame with the immunoglobulin light chain constant region.

Immunoglobulin heavy chain constant region domains include C_{H1} , C_{H2} , C_{H3} , and C_{H4} of any class of immunoglobulin heavy chain including γ , α , ϵ , μ , and δ classes (Honjo, ibid., 1983) A particularly preferred immunoglobulin heavy chain constant region domain is human C_{H1} . Immunoglobulin variable regions include V_H , V_K , or V_λ .

DNA sequences encoding immunoglobulins may be cloned from a variety of genomic or cDNA libraries known in the art. The techniques for isolating such DNA sequences using probe-based methods are conventional 5 techniques and are well known to those skilled in the art. Probes for isolating such DNA sequences may be based on published DNA sequences (see, for example, Hieter et al., Cell 22: 197-207, 1980). Alternatively, the polymerase chain reaction (PCR) method disclosed by Mullis et al. 10 (U.S. Patent No. 4,683,195) and Mullis (U.S. Patent No. 4,683,202), incorporated herein by reference may be used. The choice of library and selection of probes for the isolation of such DNA sequences is within the level of ordinary skill in the art.

15 Host cells for use in practicing the present invention include eukaryotic cells capable of being transformed or transfected with exogenous DNA and grown in culture, such as cultured mammalian and fungal cells. Fungal cells, including species of yeast (e.g., 20 Saccharomyces spp., Schizosaccharomyces spp.), or filamentous fungi (e.g., Aspergillus spp., Neurospora spp.) may be used as host cells within the present invention. Strains of the yeast Saccharomyces cerevisiae are particularly preferred.

25 Expression units for use in the present invention will generally comprise the following elements, operably linked in a 5' to 3' orientation: a transcriptional promoter, a secretory signal sequence a DNA sequence encoding nonimmunoglobulin polypeptide 30 requiring dimerization for biological activity joined to a dimerizing protein and a transcriptional terminator. The selection of suitable promoters, signal sequences and terminators will be determined by the selected host cell and will be evident to one skilled in the art and are 35 discussed more specifically below.

Suitable yeast vectors for use in the present invention include YRp7 (Struhl et al., Proc. Natl. Acad.

Sci. USA 76: 1035-1039, 1978), YEpl3 (Broach et al., Gene 8: 121-133, 1979), pJDB249 and pJDB219 (Beggs, Nature 275:104-108, 1978) and derivatives thereof. Such vectors will generally include a selectable marker, which may be
5 one of any number of genes that exhibit a dominant phenotype for which a phenotypic assay exists to enable transformants to be selected. Preferred selectable markers are those that complement host cell auxotrophy, provide antibiotic resistance or enable a cell to utilize
10 specific carbon sources, and include LEU2 (Broach et al. ibid.), URA3 (Botstein et al., Gene 8: 17, 1979), HIS3 (Struhl et al., ibid.) or POT1 (Kawasaki and Bell, EP 171,142). Other suitable selectable markers include the
15 CAT gene, which confers chloramphenicol resistance on yeast cells.

Preferred promoters for use in yeast include promoters from yeast glycolytic genes (Hitzeman et al., J. Biol. Chem. 255: 12073-12080, 1980; Alber and Kawasaki, J. Mol. Appl. Genet. 1: 419-434, 1982; Kawasaki, U.S. Patent
20 No. 4,599,311) or alcohol dehydrogenase genes (Young et al., in Genetic Engineering of Microorganisms for Chemicals, Hollaender et al., (eds.), p. 355, Plenum, New York, 1982; Ammerer, Meth. Enzymol. 101: 192-201, 1983). In this regard, particularly preferred promoters are the
25 TPI1 promoter (Kawasaki, U.S. Patent No. 4,599,311, 1986) and the ADH2-4^C promoter (Russell et al., Nature 304: 652-654, 1983 and Irani and Kilgore, described in pending, commonly assigned U.S. Patent Application Serial No. 07/183,130, which is incorporated herein by reference).
30 The expression units may also include a transcriptional terminator. A preferred transcriptional terminator is the TPI1 terminator (Alber and Kawasaki, ibid.).

In addition to yeast, proteins of the present invention can be expressed in filamentous fungi, for
35 example, strains of the fungi Aspergillus (McKnight and Upshall, described in commonly assigned U.S. Patent 4,935,349, which is incorporated herein by reference).

Examples of useful promoters include those derived from Aspergillus nidulans glycolytic genes, such as the ADH3 promoter (McKnight et al., EMBO J. 4: 2093-2099, 1985) and the tpiA promoter. An example of a suitable terminator is
5 the ADH3 terminator (McKnight et al., ibid.). The expression units utilizing such components are cloned into vectors that are capable of insertion into the chromosomal DNA of Aspergillus.

Techniques for transforming fungi are well known
10 in the literature, and have been described, for instance, by Beggs (ibid.), Hinnen et al. (Proc. Natl. Acad. Sci. USA 75: 1929-1933, 1978), Yelton et al., (Proc. Natl. Acad. Sci. USA 81: 1740-1747, 1984), and Russell (Nature 301: 167-169, 1983). The genotype of the host cell will
15 generally contain a genetic defect that is complemented by the selectable marker present on the expression vector. Choice of a particular host and selectable marker is well within the level of ordinary skill in the art.

In a preferred embodiment, a Saccharomyces cerevisiae host cell that contains a genetic deficiency in a gene required for asparagine-linked glycosylation of glycoproteins is used. Preferably, the S. cerevisiae host cell contains a genetic deficiency in the MNN9 gene (described in pending, commonly assigned U.S. Patent
25 Application Serial Nos. 116,095 and 189,547 which are incorporated by reference herein in their entirety). Most preferably, the S. cerevisiae host cell contains a disruption of the MNN9 gene. S. cerevisiae host cells having such defects may be prepared using standard
30 techniques of mutation and selection. Ballou et al. (J. Biol. Chem. 255: 5986-5991, 1980) have described the isolation of mannoprotein biosynthesis mutants that are defective in genes which affect asparagine-linked glycosylation. Briefly, mutagenized S. cerevisiae cells
35 were screened using fluoresceinated antibodies directed against the outer mannose chains present on wild-type yeast. Mutant cells that did not bind antibody were

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further characterized and were found to be defective in the addition of asparagine-linked oligosaccharide moieties. To optimize production of the heterologous proteins, it is preferred that the host strain carries a 5 mutation, such as the S. cerevisiae pep4 mutation (Jones, Genetics 85: 23-33, 1977), which results in reduced proteolytic activity.

In addition to fungal cells, cultured mammalian cells may be used as host cells within the present 10 invention. Preferred cell lines are rodent myeloma cell lines, which include p3X63Ag8 (ATCC TIB 9), FO (ATCC CRL 1646), NS-1 (ATCC TIB 18) and 210-RCY-Ag1 (Galfre et al., Nature 277: 131, 1979). A particularly preferred rodent myeloma cell line is SP2/0-Ag14 (ATCC CRL 1581). In 15 addition, a number of other cell lines may be used within the present invention, including COS-1 (ATCC CRL 1650), BHK, p363.Ag.8.653 (ATCC CRL 1580) Rat Hep I (ATCC CRL 1600), Rat Hep II (ATCC CRL 1548), TCMK (ATCC CCL 139), Human lung (ATCC CCL 75.1), Human hepatoma (ATCC HTB-52), 20 Hep G2 (ATCC HB 8065), Mouse liver (ATCC CC 29.1), 293 (ATCC CRL 1573; Graham et al., J. Gen. Virol. 36: 59-72, 1977) and DUKX cells (Urlaub and Chasin, Proc. Natl. Acad. Sci USA 77: 4216-4220, 1980) A preferred BHK cell line is the tk⁻ts13 BHK cell line (Waechter and Baserga, Proc. Natl. Acad. Sci USA 79: 1106-1110, 1982). A preferred BHK cell line is the tk⁻ts13 BHK cell line (Waechter and Baserga, Proc. Natl. Acad. Sci. USA 79: 1106-1110, 1982). 25 A tk⁻ BHK cell line is available from the American Type Culture Collection, Rockville, MD, under accession number CRL 1632. A particularly preferred tk⁻ BHK cell line is BHK 570 which is available from the American Type Culture Collection under accession number 10314.

Mammalian expression vectors for use in carrying 30 out the present invention will include a promoter capable of directing the transcription of a cloned gene or cDNA. Preferred promoters include viral promoters and cellular promoters. Preferred viral promoters include the major

late promoter from adenovirus 2 (Kaufman and Sharp, Mol. Cell. Biol. 2: 1304-13199, 1982) and the SV40 promoter (Subramani et al., Mol. Cell. Biol. 1: 854-864, 1981). Preferred cellular promoters include the mouse metallothionein 1 promoter (Palmiter et al., Science 222: 809-814, 1983) and a mouse V_K promoter (Grant et al., Nuc. Acids Res. 15: 5496, 1987). A particularly preferred promoter is a mouse V_H promoter (Loh et al., ibid.). Such expression vectors may also contain a set of RNA splice sites located downstream from the promoter and upstream from the DNA sequence encoding the peptide or protein of interest. Preferred RNA splice sites may be obtained from adenovirus and/or immunoglobulin genes. Also contained in the expression vectors is a polyadenylation signal located downstream of the coding sequence of interest. Polyadenylation signals include the early or late polyadenylation signals from SV40 (Kaufman and Sharp, ibid.), the polyadenylation signal from the adenovirus E1B region and the human growth hormone gene terminator (DeNoto et al., Nuc. Acids Res. 9: 3719-3730, 1981). A particularly preferred polyadenylation signal is the V_H gene terminator (Loh et al., ibid.). The expression vectors may include a noncoding viral leader sequence, such as the adenovirus 2 tripartite leader, located between the promoter and the RNA splice sites. Preferred vectors may also include enhancer sequences, such as the SV40 enhancer and the mouse μ enhancer (Gillies, Cell 33: 717-728, 1983). Expression vectors may also include sequences encoding the adenovirus VA RNAs.

Cloned DNA sequences may be introduced into cultured mammalian cells by, for example, calcium phosphate-mediated transfection (Wigler et al., Cell 14: 725, 1978; Corsaro and Pearson, Somatic Cell Genetics 7: 603, 1981; Graham and Van der Eb, Virology 52: 456, 1973.) Other techniques for introducing cloned DNA sequences into mammalian cells, such as electroporation (Neumann et al., EMBO J. 1: 841-845, 1982), may also be used. In order to

identify cells that have integrated the cloned DNA, a selectable marker is generally introduced into the cells along with the gene or cDNA of interest. Preferred selectable markers for use in cultured mammalian cells 5 include genes that confer resistance to drugs, such as neomycin, hygromycin, and methotrexate. The selectable marker may be an amplifiable selectable marker. A preferred amplifiable selectable marker is the DHFR gene. A particularly preferred amplifiable marker is the DHFR^r 10 cDNA (Simonsen and Levinson, Proc. Natl. Acad. Sci. USA 80: 2495-2499, 1983). Selectable markers are reviewed by Thilly (Mammalian Cell Technology, Butterworth Publishers, Stoneham, MA) and the choice of selectable markers is well within the level of ordinary skill in the art.

15 Selectable markers may be introduced into the cell on a separate plasmid at the same time as the gene of interest, or they may be introduced on the same plasmid. If on the same plasmid, the selectable marker and the gene of interest may be under the control of different 20 promoters or the same promoter, the latter arrangement producing a dicistronic message. Constructs of this type are known in the art (for example, Levinson and Simonsen, U.S. Patent No. 4,713,339). It may also be advantageous to add additional DNA, known as "carrier DNA" to the 25 mixture which is introduced into the cells.

Transfected mammalian cells are allowed to grow for a period of time, typically 1-2 days, to begin expressing the DNA sequence(s) of interest. Drug selection is then applied to select for growth of cells 30 that are expressing the selectable marker in a stable fashion. For cells that have been transfected with an amplifiable selectable marker the drug concentration may be increased in a stepwise manner to select for increased copy number of the cloned sequences, thereby increasing 35 expression levels.

Host cells containing DNA constructs of the present invention are grown in an appropriate growth

medium. As used herein, the term "appropriate growth medium" means a medium containing nutrients required for the growth of cells. Nutrients required for cell growth may include a carbon source, a nitrogen source, essential amino acids, vitamins, minerals and growth factors. The growth medium will generally select for cells containing the DNA construct by, for example, drug selection or deficiency in an essential nutrient which are complemented by the selectable marker on the DNA construct or co-transfected with the DNA construct. Yeast cells, for example, are preferably grown in a chemically defined medium, comprising a non-amino acid nitrogen source, inorganic salts, vitamins and essential amino acid supplements. The pH of the medium is preferably maintained at a pH greater than 2 and less than 8, preferably at pH 6.5. Methods for maintaining a stable pH include buffering and constant pH control, preferably through the addition of sodium hydroxide. Preferred buffering agents include succinic acid and Bis-Tris (Sigma Chemical Co., St. Louis, MO). Yeast cells having a defect in a gene required for asparagine-linked glycosylation are preferably grown in a medium containing an osmotic stabilizer. A preferred osmotic stabilizer is sorbitol supplemented into the medium at a concentration between 0.1 M and 1.5 M., preferably at 0.5 M or 1.0 M. Cultured mammalian cells are generally grown in commercially available serum-containing or serum-free media. Selection of a medium appropriate for the particular cell line used is within the level of ordinary skill in the art.

The culture medium from appropriately grown transformed or transfected host cells is separated from the cell material, and the presence of dimerized polypeptide fusions or secreted receptor analogs is demonstrated. A preferred method of detecting receptor analogs, for example, is by the binding of the receptors or portions of receptors to a receptor-specific antibody. An anti-receptor antibody may be a monoclonal or

polyclonal antibody raised against the receptor in question, for example, an anti-PDGF receptor monoclonal antibody may be used to assay for the presence of PDGF receptor analogs. Another antibody, which may be used for 5 detecting substance P-tagged peptides and proteins, is a commercially available rat anti-substance P monoclonal antibody which may be utilized to visualize peptides or proteins that are fused to the C-terminal amino acids of substance P. Ligand binding assays may also be used to 10 detect the presence of receptor analogs. In the case of PDGF receptor analogs, it is preferable to use fetal foreskin fibroblasts, which express PDGF receptors, to compete against the PDGF receptor analogs of the present invention for labeled PDGF and PDGF isoforms.

15 Assays for detection of secreted, biologically active peptide dimers and receptor analogs may include Western transfer, protein blot or colony filter. A Western transfer filter may be prepared using the method described by Towbin et al. (Proc. Natl. Acad. Sci. USA 76: 20 4350-4354, 1979). Briefly, samples are electrophoresed in a sodium dodecylsulfate polyacrylamide gel. The proteins in the gel are electrophoretically transferred to nitrocellulose paper. Protein blot filters may be prepared by filtering supernatant samples or concentrates 25 through nitrocellulose filters using, for example, a Minifold (Schleicher & Schuell, Keene, NH). Colony filters may be prepared by growing colonies on a nitrocellulose filter that has been laid across an appropriate growth medium. In this method, a solid medium 30 is preferred. The cells are allowed to grow on the filters for at least 12 hours. The cells are removed from the filters by washing with an appropriate buffer that does not remove the proteins bound to the filters. A preferred buffer comprises 25 mM Tris-base, 19 mM glycine, 35 pH 8.3, 20% methanol.

The dimerized polypeptide fusions and receptor analogs present on the Western transfer, protein blot or

colony filters may be visualized by specific antibody binding using methods known in the art. For example, Towbin et al. (*ibid.*) describe the visualization of proteins immobilized on nitrocellulose filters with a 5 specific antibody followed by a labeled second antibody, directed against the first antibody. Kits and reagents required for visualization are commercially available, for example, from Vector Laboratories, (Burlingame, CA), and Sigma Chemical Company (St. Louis, MO).

10 Secreted, biologically active dimerized polypeptide fusions and receptor analogs may be isolated from the medium of host cells grown under conditions that allow the secretion of the biologically active dimerized polypeptide fusions and receptor analogs. The cell 15 material is removed from the culture medium, and the biologically active dimerized polypeptide fusions and receptor analogs are isolated using isolation techniques known in the art. Suitable isolation techniques include precipitation and fractionation by a variety of 20 chromatographic methods, including gel filtration, ion exchange chromatography and immunoaffinity chromatography. A particularly preferred purification method is immunoaffinity chromatography using an antibody directed against the receptor analog or dimerized polypeptide 25 fusion. The antibody is preferably immobilized or attached to a solid support or substrate. A particularly preferred substrate is CNBr-activated Sepharose (Pharmacia LKB Technologies, Inc., Piscataway, NJ). By this method, the medium is combined with the antibody/substrate under 30 conditions that will allow binding to occur. The complex may be washed to remove unbound material, and the receptor analog or peptide dimer is released or eluted through the use of conditions unfavorable to complex formation. Particularly useful methods of elution include changes in 35 pH, wherein the immobilized antibody has a high affinity for the ligand at a first pH and a reduced affinity at a second (higher or lower) pH; changes in concentration of

certain chaotropic agents; or through the use of detergents.

The secreted PDGF receptor analogs of the present invention can be used within a variety of assays 5 for detecting the presence of and/or screening for native PDGF, PDGF isoforms or PDGF-like molecules. These assays will typically involve combining PDGF receptor analogs, which may be bound to a solid substrate such as polymeric microtiter plate wells, with a biological sample under 10 conditions that permit the formation of receptor/ligand complexes. Screening assays for the detection of PDGF, PDGF isoforms or PDGF-like molecules will typically involve combining soluble PDGF receptor analogs with a biological sample and incubating the mixture with a PDGF 15 isoform or mixture of PDGF isoforms bound to a solid substrate such as polymeric microtiter plates, under conditions that permit the formation of receptor/ligand complexes. Detection may be achieved through the use of a label attached to the receptor or through the use of a 20 labeled antibody which is reactive with the receptor. Alternatively, the labeled antibody may be reactive with the ligand. A wide variety of labels may be utilized, such as radionuclides, fluorophores, enzymes and luminescers. Complexes may also be detected visually, 25 i.e., in immunoprecipitation assays, which do not require the use of a label.

Secreted PDGF receptor analogs of the present invention may also be labeled with a radioisotope or other imaging agent and used for in vivo diagnostic purposes. 30 Preferred radioisotope imaging agents include iodine-125 and technetium-99, with technetium-99 being particularly preferred. Methods for producing protein-isotope conjugates are well known in the art, and are described by, for example, Eckelman et al. (U.S. Patent No. 35 4,652,440), Parker et al. (WO 87/05030) and Wilber et al. (EP 203,764). Alternatively, the secreted receptor analogs may be bound to spin label enhancers and used for

magnetic resonance (MR) imaging. Suitable spin label enhancers include stable, sterically hindered, free radical compounds such as nitroxides. Methods for labeling ligands for MR imaging are disclosed by, for 5 example, Coffman et al. (U.S. Patent No. 4,656,026). For administration, the labeled receptor analogs are combined with a pharmaceutically acceptable carrier or diluent, such as sterile saline or sterile water. Administration is preferably by bolus injection, preferably 10 intravenously. These imaging agents are particularly useful in identifying the locations of atherosclerotic plaques, PDGF-producing tumors, and receptor-bound PDGF.

The secreted PDGF receptor analogs of the present invention may also be utilized within diagnostic 15 kits. Briefly, the subject receptor analogs are preferably provided in a lyophilized form or immobilized onto the walls of a suitable container, either alone or in conjunction with antibodies capable of binding to native PDGF or selected PDGF isoform(s) or specific ligands. The 20 antibodies, which may be conjugated to a label or unconjugated, are generally included in the kits with suitable buffers, such as phosphate, stabilizers, inert proteins or the like. Generally, these materials are present in less than about 5% weight based upon the amount 25 of active receptor analog, and are usually present in an amount of at least about 0.001% weight. It may also be desirable to include an inert excipient to dilute the active ingredients. Such an excipient may be present from about 1% to 99% weight of the total composition. In 30 addition, the kits will typically include other standard reagents, instructions and, depending upon the nature of the label involved, reactants that are required to produce a detectable product. Where an antibody capable of binding to the receptor or receptor/ligand complex is 35 employed, this antibody will usually be provided in a separate vial. The antibody is typically conjugated to a label and formulated in an analogous manner with the

formulations briefly described above. The diagnostic kits, including the containers, may be produced and packaged using conventional kit manufacturing procedures.

As noted above, the secreted PDGF receptor analogs of the present invention may be utilized within methods for purifying PDGF from a variety of samples. Within a preferred method, the secreted PDGF receptor analogs are immobilized or attached to a substrate or support material, such as polymeric tubes, beads, polysaccharide particulates, polysaccharide-containing materials, polyacrylamide or other water insoluble polymeric materials. Methods for immobilization are well known in the art (Mosbach et al., U.S. Patent No. 4,415,665; Clarke et al., Meth. Enzymology 68: 436-442, 1979). A common method of immobilization is CNBr activation. Activated substrates are commercially available from a number of suppliers, including Pharmacia (Piscataway, NJ), Pierce Chemical Co. (Rockford, IL) and Bio-Rad Laboratories (Richmond, CA). A preferred substrate is CNBr-activated Sepharose (Pharmacia, Piscataway, NJ). Generally, the substrate/receptor analog complex will be in the form of a column. The sample is then combined with the immobilized receptor analog under conditions that allow binding to occur. The substrate with immobilized receptor analog is first equilibrated with a buffer solution of a composition in which the receptor analog has been previously found to bind its ligand. The sample, in the solution used for equilibration, is then applied to the substrate/receptor analog complex. Where the complex is in the form of a column, it is preferred that the sample be passed over the column two or more times to permit full binding of ligand to receptor analog. The complex is then washed with the same solution to elute unbound material. In addition, a second wash solution may be used to minimize nonspecific binding. The bound material may then be released or eluted through the use of conditions unfavorable to

complex formation. Particularly useful methods include changes in pH, wherein the immobilized receptor has a high affinity for PDGF at a first pH and reduced affinity at a second (higher or lower) pH; changes in concentration of 5 certain chaotropic agents; or through the use of detergents.

The secreted PDGF receptor analogs fused to dimerizing proteins of the present invention may be used in pharmaceutical compositions for topical or intravenous 10 application. The secreted PDGF receptor analogs of the present invention may be useful in the treatment of atherosclerosis by, for example, binding endogenous PDGF to prevent smooth muscle cell proliferation. The PDGF receptor analogs fused to dimerizing proteins are used in 15 combination with a physiologically acceptable carrier or diluent. Preferred carriers and diluents include saline and sterile water. Pharmaceutical compositions may also contain stabilizers and adjuvants. The resulting aqueous solutions may be packaged for use or filtered under 20 aseptic conditions and lyophilized, the lyophilized preparation being combined with a sterile aqueous solution prior to administration.

The following examples are offered by way of illustration and not by way of limitation.

25

EXAMPLES

Enzymes, including restriction enzymes, DNA polymerase I (Klenow fragment), T4 DNA polymerase, T4 DNA 30 ligase and T4 polynucleotide kinase, were obtained from New England Biolabs (Beverly, MA), GIBCO-BRL (Gaithersburg, MD) and Boerhinger-Mannheim Biochemicals (Indianapolis, IN) and were used as directed by the manufacturer or as described in Maniatis et al. (Molecular 35 Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, NY, 1982) and Sambrook et al. (Molecular

Cloning: A Laboratory Manual/Second Edition, Cold Spring Harbor Laboratory, NY, 1989).

Example 1

5 Cloning PDGF Receptor cDNAs

A. Cloning the PDGF β -Receptor

A cDNA encoding the PDGF β -receptor was cloned as follows. Complementary DNA (cDNA) libraries were prepared from poly(A) RNA from diploid human dermal fibroblast 10 cells, prepared by explant from a normal adult, essentially as described by Hagen et al. (Proc. Natl. Acad. Sci. USA **83**: 2412-2416, 1986). Briefly, the poly(A) RNA was primed with oligo d(T) and cloned into λ gt11 using Eco RI linkers. The random primed library was screened 15 for the presence of human PDGF receptor cDNA's using three oligonucleotide probes complementary to sequences of the mouse PDGF receptor (Yarden et al., Nature **323**: 226-232, 1986). Approximately one million phage from the random primed human fibroblast cell library were screened using 20 oligonucleotides ZC904, ZC905 and ZC906 (Table 1; Sequence ID Numbers 5, 6 and 7, respectively). Eight positive clones were identified and plaque purified. Two clones, designated RP41 and RP51, were selected for further analysis by restriction enzyme mapping and DNA sequence 25 analysis. RP51 was found to contain 356 bp of 5'-noncoding sequence, the ATG translation initiation codon and 738 bp of the amino terminal coding sequence. RP41 was found to overlap clone RP51 and contained 2649 bp encoding amino acids 43-925 of the β -receptor protein.

30

Table 1
Oligonucleotide Sequences

ZC871 (Sequence ID Number 3)

35 5' CTC TCT TCC TCA GGT AAA TGA GTG CCA GGG CCG GCA AGC CCC CGC TCC 3'

ZC872 (Sequence ID Number 4)

5' CCG GGG AGC GGG GGC TTG CCG GCC CTG GCA CTC ATT TAC CTG
AGG AAG AGA GAG CT 3'

5 ZC904 (Sequence ID Number 5)

5' CAT GGG CAC GTA ATC TAT AGA TTC ATC CTT GCT CAT ATC CAT
GTA 3'

ZC905 (Sequence ID Number 6)

10 5' TCT TGC CAG GGC ACC TGG GAC ATC TGT TCC CAC ATC ACC GG
3'

ZC906 (Sequence ID Number 7)

5' AAG CTG TCC TCT GCT TCA GCC AGA GGT CCT GGG CAG CC 3'

15

ZC1380 (Sequence ID Number 8)

5' CAT GGT CGA ATT CCT GCT GAT 3'

ZC1447 (Sequence ID Number 9)

20 5' TG GTT GTG CAG AGC TGA GGA AGA GAT GGA 3'

ZC1453 (Sequence ID Number 10)

5' AAT TCA TTA TGT TGT TGC AAG CCT TCT TGT TCC TGC TAG CTG
GTT TCG CTG TTA A 3'

25

ZC1454 (Sequence ID Number 11)

5' GAT CTT AAC AGC GAA ACC AGC TAG CAG GAA CAA GAA GGC TTG
CAA CAA CAT AAT G 3'

30

ZC1478 (Sequence ID Number 12)

5' ATC GCG AGC ATG CAG ATC TGA 3'

ZC1479 (Sequence ID Number 13)

5' AGC TTC AGA TCT GCA TGC TGC CGA T 3'

35

ZC1776 (Sequence ID Number 14)

5' AGC TGA GCG CAA ATG TTG TGT CGA GTG CCC ACC GTG CCC AGC
TTA GAA TTC T 3'

ZC1777 (Sequence ID Number 15)

5 5' CTA GAG AAT TCT AAG CTG GGC ACG GTG GGC ACT CGA CAC AAC
ATT TGC GCT C 3'

ZC1846 (Sequence ID Number 16)

10 5' GAT CGG CCA CTG TCG GTG CGC TGC ACG CTG CGC AAC GCT GTG
GGC CAG GAC ACG CAG GAG GTC ATC GTG GTG CCA CAC TCC TTG
CCC TTT AAG CA 3'

ZC1847 (Sequence ID Number 17)

15 5' AGC TTG CTT AAA GGG CAA GGA GTG TGG CAC CAC GAT GAC CTC
CTG CGT GTC CTG GCC CAC AGC GTT GCG CAG CGT GCA GCG CAC
CGA CAG TGG CC 3'

ZC1886 (Sequence ID Number 18)

20 5' CCA GTG CCA AGC TTG TCT AGA CTT ACC TTT AAA GGG CAA GGA
G 3'

ZC1892 (Sequence ID Number 19)

5' AGC TTG AGC GT 3'

25 ZC1893 (Sequence ID Number 20)

5' CTA GAC GCT CA 3'

ZC1894 (Sequence ID Number 21)

30 5' AGC TTC CAG TTC TTC GGC CTC ATG TCA GTT CTT CGG CCT CAT
GTG AT 3'

ZC1895 (Sequence ID Number 22)

5' CTA GAT CAC ATG AGG CCG AAG AAC TGA CAT GAG GCC GAA GAA
CTG GA 3'

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ZC2181 (Sequence ID Number 23)

5' AAT TCG GAT CCA CCA TGG GCA CCA GCC ACC CGG CGT TCC TGG
TGT TAG GCT GCC TGC TGA CCG GCC 3'

ZC2182 (Sequence ID Number 24)

5 5' TGA GCC TGA TCC TGT GCC AAC TGA GCC TGC CAT CGA TCC TGC
CAA ACG AGA ACG AGA AGG TTG TGC AGC TA 3'

ZC2183 (Sequence ID Number 25)

5' AAT TTA GCT GCA CAA CCT TCT CGT TCT CGT TTG GCA GGA TCG
10 ATG GCA GGC TCA GTT GGC ACA GGA TCA 3'

ZC2184 (Sequence ID Number 26)

5' GGC TCA GGC CGG TCA GCA GGC AGC CTA ACA CCA GGA ACG CCG
GGT GGC TGG TGC CCA TGG TGG ATC CG 3'

15

ZC2311 (Sequence ID Number 27)

5' TGA TCA CCA TGG CTC AAC TG 3'

ZC2351 (Sequence ID Number 28)

20 5' CGA ATT CCA C 3'

ZC2352 (Sequence ID Number 29)

5' CAT GGT GGA ATT CGA GCT 3'

25

ZC2392 (Sequence ID Number 30)

5' ACG TAA GCT TGT CTA GAC TTA CCT TCA GAA CGC AGG GTG GG
3'

The 3'-end of the cDNA was not isolated in the
30 first cloning and was subsequently isolated by screening
 6×10^5 phage of the oligo d(T)-primed cDNA library with a
630 bp Sst I-Eco RI fragment derived from the 3'-end of
clone RP41. One isolate, designated OT91, was further
analyzed by restriction enzyme mapping and DNA sequencing.
35 This clone was found to comprise the 3'-end of the
receptor coding region and 1986 bp of 3' untranslated
sequence.

Clones RP51, RP41 and OT91 were ligated together to construct a full-length cDNA encoding the entire PDGF β -receptor. RP41 was digested with Acc I and Bam HI to isolate the 2.12 kb fragment. RP51 was digested with Eco RI and Acc I to isolate the 982 bp fragment. The 2.12 kb RP41 fragment and the 982 bp RP51 fragment were joined in a three-part ligation with pUC13, which had been linearized by digestion with Eco RI and Bam HI. The resultant plasmid was designated 51/41. Plasmid 51/41 was digested with Eco RI and Bam HI to isolate the 3 kb fragment comprising the partial PDGF receptor cDNA. OT91 was digested with Bam HI and Xba I to isolate the 1.4 kb fragment containing the 3' portion of the PDGF receptor cDNA. The Eco RI-Bam HI 51/41 fragment, the Bam HI-Xba I OT91 fragment and the Eco RI-Xba I digested pUC13 were joined in a three-part ligation. The resultant plasmid was designated pR-RX1 (Figure 2).

B. Cloning the PDGF- α Receptor

A cDNA encoding to PDGF α -receptor was cloned as follows. RNA was prepared by the method of Chirgwin et al. (Biochemistry 18: 5294, 1979) and twice purified on oligo d(T) cellulose to yield poly(A)+ RNA. Complementary DNA was prepared in λ gt10 phage using a kit purchased from Invitrogen (San Diego, CA). The resulting λ phage DNA was packaged with a coat particle mixture from Stratagene Cloning Systems (La Jolla, CA), infected into E. coli strain C600 Hfl⁻ and titered.

Approximately 1.4×10^6 phage recombinants were plated to produce plaques for screening. Nitrocellulose filter lifts were prepared according to standard methods and were hybridized to a radiolabeled PDGF β -receptor DNA fragment (Gronwald et al., ibid.) comprising the 1.9 kb Fsp I-Hind III fragment that encodes the transmembrane and cytoplasmic domains of the PDGF β -receptor cDNA. Hybridization was performed for 36 hours at 42°C in a mixture containing 40% formamide, 5x SSCP (SSC containing

25 mM phosphate buffer, pH 6.5), 200 µg/ml denatured salmon sperm DNA, 3x Denhardt's, and 10% dextran sulfate. Following hybridization, the filters were washed extensively at room temperature in 2x SSC, then for 15 5 minutes at 47-48°C. Following an exposure to X-ray film, the filters were treated to increasingly stringent wash conditions followed by film recording until a final wash with 0.1x SSC at 65°C was reached. Film analysis showed that a "family" of plaques that hybridized at lower wash 10 stringency but not at the highest stringency. This "family" was selected for further analysis.

Two λ phage clones from the "family" obtained from the initial screening were subcloned into the Not I site of the pUCtype plasmid vector pBluescript SK⁺ 15 (obtained from Stratagene Cloning Systems, La Jolla, CA) and were analyzed by restriction mapping and sequence analysis. Restriction enzyme analysis of a phage clone, designated α1-1, revealed a restriction fragment pattern dissimilar from that of the PDGF β-receptor cDNA with the 20 exception of a common Bgl II-Bgl II band of approximately 160 bp. The PDGF β-receptor cDNA contains two similarly spaced Bgl II sites within the region coding for the second tyrosine kinase domain.

Restriction analysis of a second plasmid 25 subclone (designated α1-7) revealed an overlap of the 5' approximately 1.2 kb of clone α1-1, and an additional approximately 2.2 kb of sequence extending in the 5' direction. Sequence analysis revealed that the 3' end of this clone encodes the second tyrosine kinase domain, 30 which contains regions of near sequence identity to the corresponding regions in the PDGF β-receptor. The 5' end of clone α1-7 contained non-receptor sequences. Two additional α-receptor clones were obtained by probing with α1-1 sequences. Clone α1-1 was digested with Not I and Spe 35 I, and a 230 bp fragment was recovered. Clone α1-1 was also digested with Bam HI and Not I, and a 550 bp fragment was recovered. A clone that hybridized to the 230 bp

probe was designated $\alpha 5-1$. This clone contained the 5'-most coding sequence for the PDGF α -receptor. Another clone, designated $\alpha 6-3$, hybridized to the 550 bp probe and was found to contain 3' coding and non-coding sequences, 5 including the poly(A) tail.

Clone $\alpha 1-1$ was radiolabeled (^{32}P) and used to probe a northern blot (Thomas, Methods Enzymol. 100: 225-265, 1983) of the MG-63 poly(A)+ RNA used to prepare the cDNA library. A single band of approximately 6.6 kb was 10 observed. RNA prepared from receptor-positive cell lines including the human fibroblast SK4, WI-38 and 7573 cell lines; the mouse fibroblast line DI 3T3; the U2-OS human osteosarcoma cell line and baboon aortic smooth muscle cells, and RNA prepared from receptor-negative lines 15 including A431 (an epithelial cell line) and VA 13 (SV40-transformed WI-38 cells) were probed by northern format with the $\alpha 1-1$ cDNA. In all cases, the amount of the 6.6 kb band detected in these RNA correlated well with the relative levels of α -receptor detected on the respective 20 cell surfaces. The 6.6 kb RNA was not detected in RNA prepared from any tested cell line of hematopoietic origin, in agreement with a lack of PDGF α -receptor protein detected on these cell types.

Clones $\alpha 1-1$ and $\alpha 1-7$ were joined at a unique 25 Pst I site in the region encoding the transmembrane portion of the receptor. Clone $\alpha 1-1$ was digested with Xba I and Pst I and the receptor sequence fragment was recovered. Clone $\alpha 1-7$ was digested with Pst I and Bam HI and the receptor fragment was recovered. The two 30 fragments were ligated with Xba I + Bam HI-digested pIC19R (Marsh et al. Gene 32: 481-486, 1984) to construct plasmid $p\alpha 17R$ (Figure 12).

The remainder of the 5'-most α -receptor sequence was obtained from clone $\alpha 5-1$ as an Sst I-Cla I fragment. 35 This fragment was joined to the Eco RI-Sst I receptor fragment of $p\alpha 17R$ and cloned into Eco RI + C1a I-digested pBluescript SK+ plasmid to construct plasmid $p\alpha 17B$ (Figure

12). Figure 11 (Sequence ID Numbers 35 and 36) shows the nucleotide sequence and deduced amino acid sequence of the cDNA present in p α 17B.

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Example 2

Construction of a SUC2 Signal Sequence-PDGF β -Receptor Fusion

To direct the PDGF β -receptor into the yeast secretory pathway, the PDGF β -receptor cDNA was joined to a sequence encoding the Saccharomyces cerevisiae SUC2 signal sequence. Oligonucleotides ZC1453 and ZC1454 (Sequence ID Numbers 10 and 11; Table 1) were designed to form an adapter encoding the SUC2 secretory signal flanked by a 5' Eco RI adhesive end and a 3' Bgl II adhesive end. ZC1453 and ZC1454 were annealed under conditions described by Maniatis et al. (ibid.). Plasmid pR-RX1 was digested with Bgl II and Sst II to isolate the 1.7 kb fragment comprising the PDGF β -receptor coding sequence from amino acids 28 to 596. Plasmid pR-RX1 was also cut with Sst II and Hind III to isolate the 1.7 kb fragment comprising the coding sequence from amino acids 597 through the translation termination codon and 124 bp of 3' untranslated DNA. The two 1.7 kb pR-RX1 fragments and the ZC1453/ZC1454 adapter were joined with pUC19, which had been linearized by digestion with Eco RI and Hind III. The resultant plasmid, comprising the SUC2 signal sequence fused in-frame with the PDGF β -receptor cDNA, was designated pBTL10 (Figure 2).

30

Example 3

Construction of pCBS22

The BAR1 gene product, Barrier, is an exported protein that has been shown to have three domains. When used in conjunction with a first signal sequence, the third domain of Barrier protein has been shown to aid in

the secretion of proteins into the medium (MacKay et al., U.S. Patent Application Serial No. 104,316).

- The portion of the BAR1 gene encoding the third domain of Barrier was joined to a sequence encoding the C-terminal portion of substance P (subP; Munro and Pelham, EMBO J. 3: 3087-3093, 1984). The presence of the substance P amino acids on the terminus of the fusion protein allowed the protein to be detected using commercially available anti-substance P antibodies.
- 10 Plasmid pZV9 (deposited as a transformant in E. coli strain RR1, ATCC accession no. 53283), comprising the entire BAR1 coding region and its associated flanking regions, was cut with Sal I and Bam HI to isolate the 1.3 kb BAR1 fragment. This fragment was subcloned into pUC13,
- 15 which had been cut with Sal I and Bam HI, to generate the plasmid designated pZV17. Plasmid pZV17 was digested with Eco RI to remove the 3'-most 0.5 kb of the BAR1 coding region. The vector-BAR1 fragment was religated to create the plasmid designated pJH66 (Figure 3). Plasmid pJH66
- 20 was linearized with Eco RI and blunt-ended with DNA polymerase I (Klenow fragment). Kinased Bam HI linkers (5' CCG GAT CCG G 3') were added and excess linkers were removed by digestion with Bam HI before religation. The resultant plasmid was designated pSW8 (Figure 3).
- 25 Plasmid pSW81, comprising the TPI1 promoter, the BAR1 coding region fused to the coding region of the C-terminal portion of substance P (Munro and Pelham, EMBO J. 3: 3087-3093, 1984) and the TPI1 terminator, was derived from pSW8. Plasmid pSW8 was cut with Sal I and Bam HI to
- 30 isolate the 824 bp fragment encoding amino acids 252 through 526 of BAR1. Plasmid pPM2, containing the synthetic oligonucleotide sequence encoding the dimer form of the C-terminal portion of substance P (subP) in M13mp8, was obtained from Hugh Pelham (MRC Laboratory of Molecular
- 35 Biology, Cambridge, England). Plasmid pPM2 was linearized by digestion with Bam HI and Sal I and ligated with the 824 bp BAR1 fragment from pSW8. The resultant plasmid,

pSW14, was digested with Sal I and Sma I to isolate the 871 bp BAR1-substance P fragment. Plasmid pSW16, comprising a fragment of BAR1 encoding amino acids 1 through 250, was cut with Xba I and Sal I to isolate the 5 767 bp BAR1 fragment. This fragment was ligated with the 871 bp BAR1-substance P fragment in a three-part ligation with pUC18 cut with Xba I and Sma I. The resultant plasmid, designated pSW15, was digested with Xba I and Sma I to isolate the 1.64 kb BAR1-substance P fragment. The 10 ADH1 promoter was obtained from pRL029. Plasmid pRL029, comprising the ADH1 promoter and the BAR1 5' region encoding amino acids 1 to 33 in pUC18, was digested with Sph I and Xba I to isolate the 0.42 kb ADH1 promoter fragment. The TPI1 terminator (Alber and Kawasaki, *ibid.*) 15 was provided as a linearized fragment containing the TPI1 terminator and pUC18 with a Klenow-filled Xba I end and an Sph I end. This fragment was ligated with the 0.42 kb ADH1 promoter fragment and the 1.64 kb BAR1-substance P fragment in a three-part ligation to produce plasmid 20 pSW22.

The ADH1 promoter and the coding region of BAR1, from the translation initiation ATG through the Eco RV site present in pSW22, were removed by digestion with Hind III and Eco RV. The 3.9 kb vector fragment, comprising 25 the 401 bp between the Eco RV and the Eco RI sites of the BAR1 gene fused to subP and the TPI1 terminator, was isolated by gel electrophoresis. Oligonucleotide ZC1478 (Sequence ID Number 12; Table 1) was kinased and annealed with oligonucleotide ZC1479 (Sequence ID Number 13; Table 30 1) using conditions described by Maniatis et al. (*ibid.*). The annealed oligonucleotides formed an adapter comprising a Hind III adhesive end and a polylinker encoding Bgl II, Sph I, Nru I and Eco RV restriction sites. The ZC1479/ZC1478 adapter was ligated with the gel-purified 35 pSW22 fragment. The resultant plasmid was designated pCBS22 (Figure 3).

Example 4

Construction of pBTL13

In order to enhance the secretion of the PDGF β -receptor and to facilitate the identification of the secreted protein, a sequence encoding the third domain of BAR1 fused to the C-terminal amino acids of substance P was fused in frame with the 5' 1240 bp of the PDGF β -receptor. Plasmid pBTL10 (Example 2) was digested with Sph I and Sst I to isolate the 4 kb fragment comprising the SUC2 signal sequence, a portion of the PDGF β -receptor cDNA and the pUC19 vector sequences. Plasmid pCBS22 was digested with Sph I and Sst I to isolate the 1.2 kb fragment comprising the BAR1-subP fusion and the TPII terminator. These two fragments were ligated, and the resultant plasmid was designated pBTL13 (Figure 4).

Example 5Construction of an Expression Vector Encoding the Entire
20 PDGF β -Receptor

The entire PDGF β -receptor was directed into the secretory pathway by fusing a SUC2 signal sequence to the 5' end of the PDGF β -receptor coding sequence. This 25 fusion was placed behind the TPII promoter and inserted into the vector YEp13 for expression in yeast.

The TPII promoter was obtained from plasmid pTPIC10 (Alber and Kawasaki, J. Mol. Appl. Genet. 1: 410-434, 1982), and plasmid pFATPOT (Kawasaki and Bell, EP 30 171,142; ATCC 20699). Plasmid pTPIC10 was cut at the unique Kpn I site, the TPII coding region was removed with Bal-31 exonuclease, and an Eco RI linker (sequence: GGA ATT CC) was added to the 3' end of the promoter. Digestion with Bgl II and Eco RI yielded a TPII promoter 35 fragment having Bgl II and Eco RI sticky ends. This fragment was then joined to plasmid YRp7' (Stinchcomb et al., Nature 282: 39-43, 1979) that had been cut with Bgl

II and Eco RI (partial). The resulting plasmid, TE32, was cleaved with Eco RI (partial) and Bam HI to remove a portion of the tetracycline resistance gene. The linearized plasmid was then recircularized by the addition
5 of an Eco RI-Bam HI linker to produce plasmid TEA32. Plasmid TEA32 was digested with Bgl II and Eco RI, and the 900 bp partial TPI1 promoter fragment was gel-purified. Plasmid pIC19H (Marsh et al., Gene 32:481-486, 1984) was cut with Bgl II and Eco RI and the vector fragment was gel
10 purified. The TPI1 promoter fragment was then ligated to the linearized pIC19H and the mixture was used to transform E. coli RR1. Plasmid DNA was prepared and screened for the presence of a ~900 bp Bgl II-Eco RI fragment. A correct plasmid was selected and designated
15 pICTPIP.

The TPI1 promoter was then subcloned to place convenient restriction sites at its ends. Plasmid pIC7 (Marsh et al., *ibid.*) was digested with Eco RI, the fragment ends were blunted with DNA polymerase I (Klenow
20 fragment), and the linear DNA was recircularized using T4 DNA ligase. The resulting plasmid was used to transform E. coli RR1. Plasmid DNA was prepared from the transformants and was screened for the loss of the Eco RI site. A plasmid having the correct restriction pattern
25 was designated pIC7RI*. Plasmid pIC7RI* was digested with Hind III and Nar I, and the 2500 bp fragment was gel-purified. The partial TPI1 promoter fragment (ca. 900 bp) was removed from pICTPIP using Nar I and Sph I and was gel-purified. The remainder of the TPI1 promoter was
30 obtained from plasmid pFATPOT by digesting the plasmid with Sph I and Hind III, and a 1750 bp fragment, which included a portion of the TPI1 promoter fragment from pICTPIP, and the fragment from pFATPOT were then combined in a triple ligation to produce pMVR1 (Figure 2).

35 The TPI1 promoter was then joined to the SUC2-PDGF β -receptor fusion. Plasmid pBTL10 (Example 2) was digested with Eco RI and Hind III to isolate the 3.4 kb

fragment comprising the SUC2 signal sequence and the entire PDGF β -receptor coding region. Plasmid pMVR1 was digested with Bgl II and Eco RI to isolate the 0.9 kb TPI1 promoter fragment. The TPI1 promoter fragment and the 5 fragment derived from pBTL10 were joined with YEp13, which had been linearized by digestion with Bam HI and Hind III, in a three-part ligation. The resultant plasmid was designated pBTL12 (Figure 2).

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Example 6

Construction of an Expression Vector Encoding the 5' Extracellular Portion of the PDGF β -Receptor

The extracellular portion of the PDGF β -receptor 15 was directed into the secretory pathway by fusing the coding sequence to the SUC2 signal sequence. This fusion was placed in an expression vector behind the TPI1 promoter. Plasmid pBTL10 (Example 2) was digested with Eco RI and Sph I to isolate the approximately 1.3 kb 20 fragment comprising the SUC2 signal sequence and the PDGF β -receptor extracellular domain coding sequence. Plasmid pMVR1 (Example 5) was digested with Bgl II and Eco RI to isolate the 0.9 kb TPI1 promoter fragment. The TPI1 promoter fragment was joined with the fragment derived 25 from pBTL10 and YEp13, which had been linearized by digestion with Bam HI and Sph I, in a three-part ligation. The resultant plasmid was designated pBTL11 (Figure 2).

Example 7

30 Construction of Yeast Expression Vectors pBTL14 and pBTL15, and The Expression of PDGF β -Receptor-BAR1-subP Fusions

A. Construction of pBTL14

35 The SUC2-PDGF β -R fusion was joined with the third domain of BAR1 to enhance the secretion of the receptor, and the expression unit was cloned into a

derivative of YEpl3 termed pJH50. YEpl3 was modified to destroy the Sal I site near the LEU2 gene. This was achieved by partially digesting YEpl3 with Sal I followed by a complete digestion with Xho I. The 2.0 kb Xho I-Sal
5 I fragment comprising the LEU2 gene and the 8.0 kb linear YEpl3 vector fragment were isolated and ligated together. The ligation mixture was transformed into E. coli strain RR1. — DNA was prepared from the transformants and was analyzed by digestion with Sal I and Xho I. A clone was
10 isolated which showed a single Sal I site and an inactive Xho I site indicating that the LEU2 fragment had inserted in the opposite orientation relative to the parent plasmid YEpl3. The plasmid was designated pJH50.

Referring to Figure 4, plasmid pBTL12 (Example
15 5) was digested with Sal I and Pst I to isolate the 2.15 kb fragment comprising 270 bp of YEpl3 vector sequence, the TPI1 promoter, the SUC2 signal sequence, and 927 bp of PDGF β -receptor cDNA. Plasmid pBTL13 (Example 4) was digested with Pst I and Hind III to isolate the 1.48 kb
20 fragment comprising 313 bp of PDGF β -receptor cDNA, the BAR1-subP fusion and the TPI1 terminator. The fragments derived from pBTL12 and pBTL13 were joined with pJH50, which had been linearized by digestion with Hind III and Sal I, in a three-part ligation. The resultant plasmid
25 was designated pBTL14.

B. Construction of pBTL15

Referring to Figure 5, a yeast expression vector was constructed comprising the TPI1 promoter, the SUC2
30 signal sequence, 1.45 kb of PDGF β -receptor cDNA sequence fused to the BAR1-subP fusion and the TPI1 terminator. Plasmid pBTL12 (Example 5) was digested with Sal I and Fsp I to isolate the 2.7 kb fragment comprising the TPI1 promoter, the SUC2 signal sequence, the PDGF β -R coding
35 sequence, and 270 bp of YEpl3 vector sequence. Plasmid pBTL13 (Example 4) was digested with Nru I and Hind III to isolate the 1.4 kb fragment comprising the BAR1-subP

fusion, the TPI1 terminator and 209 bp of 3' PDGF β -receptor cDNA sequence. The fragments derived from pBTL12 and pBTL13 were joined in a three-part ligation with pJH50, which had been linearized by digestion with Hind III and Sal I. The resultant plasmid was designated pBTL15.

c. Expression of PDGF β -R-subP fusions from pBTL14 and pBTL15

Yeast expression vectors pBTL14 and pBTL15 were transformed into Saccharomyces cerevisiae strains ZY100 (MATA leu2-3,112 ade2-101 suc2-Δ9 gal2 pep4::TPI1prom-CAT) and ZY400 (MATA leu2-3,112 ade2-101 suc2-Δ9 gal2 pep4::TPI1prom-CAT Δmnn9::URA3). Transformations were carried out using the method essentially described by Beggs (ibid.). Transformants were selected for their ability to grow on -LEUDS (Table 2).

Table 2
Media Recipes

-LeuThrTrp Amino Acid Mixture

4 g	adenine
3 g	L-arginine
25 5 g	L-aspartic acid
2 g	L-histidine free base
6 g	L-isoleucine
4 g	L-lysine-mono hydrochloride
2 g	L-methionine
30 6 g	L-phenylalanine
5 g	L-serine
5 g	L-tyrosine
4 g	uracil
6 g	L-valine

35

Mix all the ingredients and grind with a mortar and pestle until the mixture is finely ground.

-LEUDS

20 g glucose
 6.7 g Yeast Nitrogen Base without amino acids (DIFCO
 5 Laboratories Detroit, MI)
 0.6 g -LeuThrTrp Amino Acid Mixture
 182.2 g sorbitol
 18 g Agar

10 Mix all the ingredients in distilled water. Add distilled water to a final volume of 1 liter. Autoclave 15 minutes. After autoclaving add 150 mg L-threonine and 40 mg L-tryptophan. Pour plates and allow to solidify.

15 -LEUDS + sodium succinate, pH 6.5

20 g Yeast Nitrogen Base without amino acids
 0.6 g -LeuTrpThr Amino Acid Mixture
 182.2 g sorbitol
 11.8 g succinic acid

20 Mix all ingredients in distilled water to a final volume of 1 liter. Adjust the pH of the solution to pH 6.5. Autoclave 15 minutes. After autoclaving add 150 mg L-threonine and 40 mg L-tryptophan.

25

Fermentation Medium

7 g/l yeast nitrogen base without amino acids or ammonium sulfate (DIFCO Laboratories)
 0.6 g/l ammonium sulfate
 30 0.5 M sorbitol
 0.39 g/l adenine sulfate
 0.01% polypropylene glycol

Mix all ingredients in distilled water.
 35 Autoclave 15 minutes. Add 80 ml 50% glucose for each liter of medium.

Super Synthetic -LEUD, pH 6.5 (liquid or solid medium)

- 6.7 g Yeast Nitrogen Base without amino acids or ammonium sulfate (DIFCO)
 6 g ammonium sulfate
 5 160 g adenine
 0.6 g -LeuThrTrp Amino Acid Mixture
 20 g glucose
 11.8g succinic acid
- 10 Mix all ingredients and add distilled water to a final volume of 800 ml. Adjust the pH of the solution to pH 6.4. Autoclave 15 minutes. For solid medium, add 18 g agar before autoclaving, autoclave and pour plates.

15 Super Synthetic-LEUDS, pH 6.4 (liquid or solid medium)

Use the same recipe as Super Synthetic -LEUD, pH 6.4, but add 182.2 g sorbitol before autoclaving.

YEPD

- 20 20 g glucose
 20 g Bacto Peptone (DIFCO Laboratories)
 10 g Bacto Yeast Extract (DIFCO Laboratories)
 18 g agar
 4 ml adenine 1%
 25 8 ml 1% L-leucine

Mix all ingredients in distilled water, and bring to a final volume of 1 liter. Autoclave 25 minutes and pour plates.

30

The transformants were assayed for binding to an anti-PDGF β -receptor monoclonal antibody (PR7212) or an anti-substance P antibody by protein blot assay.

- 35 ZY100[pBTL14] and ZY100[pBTL15] transformants were grown overnight at 30°C in 5 ml Super Synthetic -LEUD, pH 6.4 (Table 2). ZY400[pBTL14] and ZY400[pBTL15] transformants

were grown overnight at 30°C in 5 ml Super Synthetic-LEUDS, pH 6.4 (Table 2). The cultures were pelleted by centrifugation and the supernatants were assayed for the presence of secreted PDGF β -receptor analogs by protein blot assay using methods described in Example 18. Results of assays using PR7212 are shown in Table 3.

TABLE 3
Results of a protein blot probed with PR7212

10

Transformant:

ZY100[pBTL14]	+
ZY400[pBTL14]	++
ZY100[pBTL15]	+
15 ZY400[pBTL15]	+

20

Example 8

Construction of a SUC2-PDGF β -R Fusion Comprising the
Complete PDGF β -R Extracellular Domain

A. Construction of pBTL22

The PDGF β -R coding sequence present in pBTL10 was modified to delete the coding region 3' to the extracellular PDGF β -R domain. As shown in Figure 6, 25 plasmid pBTL10 was digested with Sph I and Bam HI and with Sph I and Sst II to isolate the 4.77 kb fragment and the 466 bp fragment, respectively. The 466 bp fragment was then digested with Sau 3A to isolate the 0.17 kb fragment. The 0.17 kb fragment and the 4.77 kb were joined by 30 ligation. The resultant plasmid was designated pBTL21.

Plasmid pBTL21, containing a Bam HI site that was regenerated by the ligation of the Bam HI and Sau 3A sites, was digested with Hind III and Bam HI to isolate the 4.2 kb fragment. Synthetic oligonucleotides ZC1846 35 (Sequence ID Number 16; Table 1) and ZC1847 (Sequence ID Number 17; Table 1) were designed to form an adapter encoding the PDGF β -R from the Sau 3A site after bp 1856

(Figure 1; (Sequence ID Number 1)) to the end of the extracellular domain at 1958 bp (Figure 1; Sequence ID Number 1), having a 5' Bam HI adhesive end that destroys the Bam HI site and a 3' Hind III adhesive end.

5 Oligonucleotides ZC1846 and ZC1847 were annealed under conditions described by Maniatis et. al. (*ibid.*). The 4.2 pBTL21 fragment and the ZC1846/ZC1847 adapter were joined by ligation. The resultant plasmid, designated pBTL22, comprises the SUC2 signal sequence fused in proper reading

10 frame to the extracellular domain of PDGF β -R in the vector pUC19 (Figure 6).

B. Construction of pBTL28

An in-frame translation stop codon was inserted

15 immediately after the coding region of the PDGF β -R in pBTL22 using oligonucleotides ZC1892 (Sequence ID Number 19; Table 1) and ZC1893 (Sequence ID Number 20; Table 1). These oligonucleotides were designed to form an adapter encoding a stop codon in-frame with the PDGF β -R coding

20 sequence from pBTL22 flanked by a 5' Hind III adhesive end and a 3' Xba I adhesive end. Plasmid pBTL22 was digested with Eco RI and Hind III to isolate the 1.6 kb SUC2-PDGF β -R fragment. Plasmid pMVR1 was digested with Eco RI and Xba I to isolate the 3.68 kb fragment comprising the TPI1

25 promoter, pIC7RI* vector sequences and the TPI1 terminator. Oligonucleotides ZC1892 and ZC1893 were annealed to form a Hind III-Xba I adapter. The 1.6 kb SUC2-PDGF β -R fragment, the 3.86 kb pMVR1 fragment and the ZC1892/ZC1893 adapter were joined in a three-part

30 ligation. The resultant plasmid was designated pBTL27.

The expression unit present in pBTL27 was inserted into the yeast expression vector pJH50 by first digesting pJH50 with Bam HI and Sal I to isolate the 10.3 kb vector fragment. Plasmid pBTL27 was digested with Bgl II and Eco RI and with Xho I and Eco RI to isolate the 0.9 kb TPI1 promoter fragment and the 1.65 kb fragment, respectively. The 10.3 kb pJH50 vector fragment, the 0.9

kb TPI1 promoter fragment and 1.65 kb fragment were joined in a three-part ligation. The resultant plasmid was designated pBTL28.

5 C. Construction of Plasmid pBTL30

The PDGF β -R coding sequence present in plasmid pBTL22 was modified to encode the twelve C-terminal amino acids of substance P and an in-frame stop codon. Plasmid pBTL22 was digested with Eco RI and Hind III to isolate 10 the 1.6 kb SUC2-PDGF β -R fragment. Plasmid pMVR1 was digested with Eco RI and Xba I to isolate the 3.68 kb fragment comprising the TPI1 promoter, pIC7RI* and the TPI1 terminator. Synthetic oligonucleotides ZC1894 (Sequence ID Number 21; Table 1) and ZC1895 (Sequence ID 15 Number 22; Table 1) were annealed to form an adapter containing the codons for the twelve C-terminal amino acids of substance P followed by an in-frame stop codon and flanked on the 5' end with a Hind III adhesive end and on the 3' end with an Xba I adhesive end. The 20 ZC1894/ZC1895 adapter, the 1.6 kb SUC2-PDGF β -R fragment and the pMVR1 fragment were joined in a three-part ligation. The resultant plasmid, designated pBTL29, was digested with Eco RI and Xho I to isolate the 1.69 kb SUC2-PDGF β -R-subP-TPI1 terminator fragment. Plasmid 25 pBTL27 was digested with Bgl II and Eco RI to isolate the 0.9 kb TPI1 promoter fragment. Plasmid pJH50 was digested with Bam HI and Sal I to isolate the 10.3 kb vector fragment. The 1.69 kb pBTL29 fragment, the 0.9 kb TPI1 promoter fragment and the 10.3 kb vector fragment were 30 joined in a three-part ligation. The resulting plasmid was designated pBTL30.

Example 9

Construction and Expression of a SUC2-PDGF β -R-IgG Hinge

35 Expression Vector

An expression unit comprising the TPI1 promoter, the SUC2 signal sequence, the PDGF β -R extracellular

domain, an immunoglobulin hinge region and the TPI1 terminator was constructed. Plasmid pBTL22 was digested with Eco RI and Hind III to isolate the 1.56 kb fragment. Plasmid pMVR1 was digested with Eco RI and Xba I to 5 isolate the 3.7 kb fragment, comprising the TPI1 promoter, pIC7RI* vector sequences and the TPI1 terminator. Oligonucleotides ZC1776 (Sequence ID Number 14; Table 1) and ZC1777 (Sequence ID Number 15; Table 1) were designed to form, when annealed, an adapter encoding an 10 immunoglobulin hinge region with a 5' Hind III adhesive end and a 3' Xba I adhesive end. Oligonucleotides ZC1776 and ZC1777 were annealed under conditions described by Maniatis et al. (ibid.). The 1.56 kb pBTL22 fragment, the 3.7 kb fragment and the ZC1776/ZC1777 adapter were joined 15 in a three-part ligation, resulting in plasmid pBTL24.

The expression unit of pBTL24, comprising the TPI1 promoter, SUC2 signal sequence, PDGF β -R extracellular domain sequence, hinge region sequence, and TPI1 terminator, was inserted into pJH50. Plasmid pBTL24 was 20 digested with Xho I and Hind III to isolate the 2.4 kb expression unit. Plasmid pJH50 was digested with Hind III and Sal I to isolate the 9.95 kb fragment. The 2.4 kb pBTL24 fragment and 9.95 kb pJH50 vector fragment were joined by ligation. The resultant plasmid was designated 25 pBTL25.

Plasmid pBTL25 was transformed into Saccharomyces cerevisiae strain ZY400 using the method essentially described by Beggs (ibid.). Transformants were selected for their ability to grow on -LEUDS (Table 30 2). The transformants were tested for their ability to bind the anti-PDGFB-R monoclonal antibody PR7212 using the colony assay method described in Example 18. Plasmid pBTL25 transformants were patched onto nitrocellulose filters that had been wetted and supported by YEPD solid 35 medium. Antibody PR7212 was found to bind to the PDGFB-R-IgG hinge fusion secreted by ZY400[pBTL25] transformants.

Example 10

Construction and Expression of a SUC2 signal sequence-PDGF β -R Extracellular Domain-SUC2 Fusion

As shown in Figure 6, an expression unit comprising the TPI1 promoter, SUC2 signal sequence, PDGF β -R extracellular domain sequence, and SUC2 coding sequence was constructed as follows. Plasmid pBTL22 was digested with Eco RI and Hind III to isolate the 1.6 kb SUC2-PDGF β -R fragment. Plasmid pMVR1 was digested with Bgl II and Eco RI to isolate the 0.9 kb TPI1 promoter fragment. The SUC2 coding region was obtained from pJH40. Plasmid pJH40 was constructed by inserting the 2.0 kb Hind III-Hind III SUC2 fragment from PRB58 (Carlson et al., Cell 28:145-154, 1982) into the Hind III site of pUC19 followed by the destruction of the Hind III site 3' to the coding region. Plasmid pJH40 was digested with Hind III and Sal I to isolate the 2.0 kb SUC2 coding sequence. Plasmid pJH50 was digested with Sal I and Bam HI to isolate the 10.3 kb vector fragment. The 0.9 kb Bgl II-Eco RI TPI1 promoter fragment, the 1.6 kb Eco RI-Hind III SUC2-PDGF β -R, the 2.0 kb Hind III-Sal I SUC2 fragment and the 10.3 kb Bam HI-Sal I vector fragment were joined in a four-part ligation. The resultant plasmid was designated pBTL26 (Figure 6).

Plasmid pBTL26 was transformed into Saccharomyces cerevisiae strain ZY400 using the method essentially described by Beggs (*ibid.*). Transformants were selected for their ability to grow on -LEUDS (Table 2). ZY400 transformants (ZY400[pBTL26]) were assayed by protein blot (Example 18), colony blot (Example 18) and competition assay.

Protein blot assays were carried out on ZY400[pBTL26] and ZY400[pJH50] (control) transformants that had been grown in flasks. Two hundred-fifty microliters of a 5 ml overnight cultures of ZY400[pBTL26] and ZY400 [pJH50] in -LEUDS + sodium succinate, pH 6.5 (Table 2) were inoculated into 50 ml of -LEUDS + sodium succinate, pH 6.5. The cultures were incubated for 35

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hours in an airbath shaker at 30°C. The culture supernatants were harvested by centrifugation. The culture supernatants were assayed as described in Example 18 and were found to bind PR7212 antibody.

5 Colony assays were carried out on ZY400[pBTL26] transformants. ZY400[pBTL26] transformants were patched onto wetted nitrocellulose filters that were supported on a YEPD plate. The colony assay carried out as described in Example 8.A. showed that ZY400[pBTL26] antibodies bound
10 PR7212 antibodies.

Competition binding assays were carried out on ZY400[pBTL26] and ZY400[pJH50] transformants. The transformants were grown in two liters of fermentation medium (Table 2) in a New Brunswick Bioflo2 fermentor (New 15 Brunswick, Philadelphia, PA) with continuous pH control at pH 6.4. The cultures were adjusted to pH 7.5 immediately prior to harvesting. Culture supernatants were concentrated in an Amicon concentrator (Amicon, San Francisco, CA) using an Amicon 10⁴ mw spiral filter 20 cartridge. The concentrated supernatants were further concentrated using Amicon Centriprep 10's. Fifteen milliliters of the concentrated supernatant samples were added to the Centripreps, and the Centripreps were spun in a Beckman GRP centrifuge (Beckman Instruments Inc., 25 Carlsbad, CA) at setting 5 for a total of 60 minutes. The concentrates were removed from the Centripreps and were assayed in the competition assay.

The competition binding assay measured the amount of ¹²⁵I-PDGF left to bind to fetal foreskin 30 fibroblast cells after preincubation with the concentrate containing the PDGF β -R-SUC2 fusion protein. PDGF-AA and PDGF-AB were iodinated using the Iodopead method (Pierce Chemical). PDGF-BB_{Tyr} was iodinated and purified as described in Example 18.F. The concentrate was serially 35 diluted in binding medium (Table 4). The dilutions were mixed with 0.5 ng of iodinated PDGF-AA, PDGF-BB_{Tyr} or PDGF-AB, and the mixtures were incubated for two hours at

room temperature. Three hundred micrograms of unlabeled PDGF-BB was added to each sample mixture. The sample mixtures were added to 24-well plates containing confluent fetal foreskin fibroblast cells (AG1523, available from 5 the Human Genetic Mutant Cell Repository, Camden, NJ). The cells were incubated with the mixture for four hours at 4°C. The supernatants were aspirated from the wells, and the wells were rinsed three times with phosphate buffered saline that was held at 4°C (PBS; Sigma, St. 10 Louis, Mo.). Five hundred microliters of PBS + 1% NP-40 was added to each well, and the plates were shaken on a platform shaker for five minutes. The cells were harvested and the amount of iodinated PDGF was determined. The results of the competition binding assay showed that 15 the PDGF β -R-SUC2 fusion protein was able to competitively bind all three isoforms of PDGF.

The PDGF β -R produced from ZY400 [pBTL26] transformants was tested for cross reactivity to fibroblast growth factor (FGF) and transforming growth 20 factor- β (TGF- β) using the competition assay essentially described above. Supernatant concentrates from ZY400[pBTL26] and ZY400[JH50] (control) transformants were serially diluted in binding medium (Table 4). The dilutions were mixed with 7.9 ng of iodinated FGF or 14 ng 25 of iodinated TGF- β , and the mixtures were incubated for two hours at room temperature. Fourteen micrograms of unlabeled FGF was added to each mixture containing labeled FGF, and 7 μ g of unlabeled TGF- β was added to each mixture containing labeled TGF- β . The sample mixtures were added 30 to 24-well plates containing confluent human dermal fibroblast cells. (Human dermal fibroblast cells express both FGF receptors and TGF β receptors.) The cells were incubated with the mixtures for four hours at 4°C. Five hundred microliters of PBS + 1% NP-40 was added to each 35 well, and the plates were shaken on a platform shaker for five minutes. The cells were harvested and the amount of iodinated FGF or TGF- β bound to the cells was determined.

The results of these assays showed that the PDGF β -R-SUC2 fusion protein did not cross react with FGF or TGF- β .

Table 4
5 Reagent Recipes

Binding Medium

500 ml	Ham's F-12 medium
12 ml	1M HEPES, pH 7.4
10 5 ml	100x PSN (Penicillin/Streptomycin/Neomycin, Gibco)
— 1 g	rabbit serum albumin

Western Transfer Buffer

15	25 mM Tris, pH 8.3
	19 mM glycine, pH 8.3
	20% methanol

Western Buffer A

20	50 ml 1 M Tris, pH 7.4
	20 ml 0.25 mM EDTA, pH 7.0
	5 ml 10% NP-40
	37.5 ml 4 M NaCl
25	2.5 g gelatin

The Tris, EDTA, NP-40 and NaCl were diluted to a final volume of one liter with distilled water. The gelatin was added to 300 ml of this solution and the solution was heated in a microwave until the gelatin was 30 in solution. The gelatin solution was added back to the remainder of the first solution and stirred at 4°C until cool. The buffer was stored at 4°C.

Western Buffer B

35	50 ml 1 M Tris, pH 7.4
	20 ml 0.25 M EDTA, pH 7.0
	5 ml 10% NP-40

58.4 g NaCl
2.5 g gelatin
4 g N-lauroyl sarcosine

5 The Tris, EDTA, NP-40, and the NaCl were mixed
and diluted to a final volume of one liter. The gelatin
was added to 300 ml of this solution and heated in a
microwave until the gelatin was in solution. The gelatin
solution was added back to the original solution and the
10 N-lauroyl sarcosine was added. The final mixture was
stirred at 4°C until the solids were completely dissolved.
This buffer was stored at 4°C.

2x Loading Buffer

15 36 ml 0.5 M Tris-HCl, pH 6.8
 16 ml glycerol
 16 ml 20% SDS
 4 ml 0.5% Bromphenol Blue in 0.5 M Tris-HCl, pH 6.8

20 Mix all ingredients. Immediately before use,
add 100 μ l β -mercaptoethanol to each 900 μ l dye mix

Example 11

Construction and Expression of PDGF Receptor Analogs From BHK cells

A. Construction of pBTL114 and pBTL115

The portions of the PDGF β -receptor extracellular domain present in pBTL14 and pBTL15 were placed in a mammalian expression vector. Plasmids pBTL14 and pBTL15 were digested with Eco RI to isolate the 1695 bp and 1905 bp SUC2 signal-PDGF β -R-BAR1 fragments. The 1695 bp fragment and the 1905 bp fragment were each ligated to Zem229R that had been linearized by digestion with Eco RI.

The vector Zem229R was constructed as shown in Figure 10 from Zem229. Plasmid Zem229 is a pUC18-based

expression vector containing a unique Bam HI site for insertion of cloned DNA between the mouse metallothionein-1 promoter and SV40 transcription terminator and an expression unit containing the SV40 early promoter, mouse dihydrofolate reductase gene, and SV40 transcription terminator. Zem229 was modified to delete the Eco RI sites flanking the Bam HI cloning site and to replace the Bam HI site with a single Eco RI cloning site. The plasmid was partially digested with Eco RI, treated with DNA polymerase I (Klenow fragment) and dNTPs, and religated. Digestion of the plasmid with Bam HI followed by ligation of the linearized plasmid with a Bam HI-Eco I adapter resulted in a unique Eco RI cloning site. The resultant plasmid was designated Zem229R.

The ligation mixtures were transformed into E. coli strain RR1. Plasmid DNA was prepared and the plasmids were subjected to restriction enzyme analysis. A plasmid having the 1695 bp pBTL14 fragment inserted into Zem229R in the correct orientation was designated pBTL114 (Figure 9). A plasmid having the 1905 bp pBTL15 fragment inserted into Zem229R in the correct orientation was designated pBTL115 (Figure 9).

B. Expression of secreted PDGF β -receptor analogs in tk⁻ ts13 BHK cells

Plasmids pBTL114 and pBTL115 were each transfected into tk⁻ts13 cells using calcium phosphate precipitation (essentially as described by Graham and van der Eb, J. Gen. Virol. 36: 59-72, 1977). The transfected cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, 1x PSN antibiotic mix (Gibco 600-5640), 2.0 mM L-glutamine. The cells were selected in 250 nM methotrexate (MTX) for 14 days, and the resulting colonies were screened by the immunofilter assay (McCracken and Brown, Biotechniques, 82-87, March/April 1984). Plates were rinsed with PBS or No Serum medium (DMEM plus 1x PSN antibiotic mix). Teflon® mesh (Spectrum

Medical Industries, Los Angeles, CA) was then placed over the cells. Nitrocellulose filters were wetted with PBS or No Serum medium, as appropriate, and placed over the mesh. After six hours incubation at 37°C, filters were removed 5 and placed in Wester buffer A (Table 4) overnight at room temperature. The filters were developed using the antibody PR7212 and the procedure described in Example 8. The filters showed that conditioned media from pBTL114-transfected and pBTL115-transfected BHK cells bound the 10 PR7212 antibody indicating the presence of biologically active secreted PDGF β -R.

Example 12

15 Expression of PDGF β -Receptor Analogs in Cultured Mouse Myeloma Cells

A. Construction of pIC μ PRES

The immunoglobulin μ heavy chain promoter and enhancer were subcloned into pIC19H to provide a unique 20 Hind III site 3' to the promoter. Plasmid p μ (Grosschedl and Baltimore, Cell 41: 885-897, 1985) was digested with Sal I and Eco RI to isolate the 3.1 kb fragment comprising the μ promoter. Plasmid pIC19H was linearized by digestion with Eco RI and Xho I. The μ promoter fragment 25 and the linearized pIC19H vector fragment were joined by ligation. The resultant plasmid, designated pIC μ 3, was digested with Ava II to isolate the 700 bp μ promoter fragment. The 700 bp fragment was blunt-ended by treatment with DNA polymerase I (Klenow fragment) and 30 deoxynucleotide triphosphates. Plasmid pIC19H was linearized by digestion with Xho I, and the adhesive ends were filled in by treatment with DNA polymerase I (Klenow fragment) and deoxynucleotide triphosphates. The blunt-ended Ava II fragment was ligated with the blunt-ended, 35 linearized pIC19H, and the ligation mixture was transformed into E. coli JM83. Plasmid DNA was prepared from the transformants and was analyzed by restriction

- digest. A plasmid with a Bgl II site 5' to the promoter was designated pIC μ PR1(-). Plasmid pIC μ PR1(-) was digested with Hind III and Bgl II to isolate the 700 bp μ promoter fragment. Plasmid pIC19R was linearized by digestion with 5 Hind III and Bam HI. The 700 bp promoter fragment was joined with the linearized pIC19R by ligation. The resultant plasmid, designated pIC μ PR7, comprised the μ promoter with an unique Sma I site 5' to the promoter and a unique Hind III site 3' to the promoter.
- 10 The immunoglobulin heavy chain μ enhancer (Gillies et al., Cell 33: 717-728, 1983) was inserted into the unique Sma I site to generate plasmid pIC μ PRE8. Plasmid pJ4 (obtained from F. Blattner, Univ. Wisconsin, Madison, Wisconsin), comprising the 1.5 kb Hind III-Eco RI 15 μ enhancer fragment in the vector pAT153 (Amersham, Arlington Heights, IL), was digested with Hind III and Eco RI to isolate the 1.5 kb enhancer fragment. The adhesive ends of the enhancer fragment were filled in by treatment with T4 DNA polymerase and deoxynucleotide triphosphates.
- 20 The blunt-ended fragment and pIC μ PR7, which had been linearized by digestion with Sma I, were joined by ligation. The ligation mixture was transformed into E. coli RR1. Plasmid DNA was prepared from the transformants, and the plasmids were analyzed by 25 restriction digests. A plasmid comprising the μ enhancer and the μ promoter was designated pIC μ PRE8 (Figure 7).

B. Construction of pSDL114

- The DNA sequence encoding the extracellular 30 domain of the PDGF β -receptor was joined with the DNA sequence encoding the human immunoglobulin light chain constant region. The PDGF β -receptor extracellular domain was obtained from mpBTL22, which comprised the Eco RI-Hind III fragment from pBTL22 (Example 8.A.) cloned into Eco 35 RI-Hind III cut M13mp18. Single stranded DNA was prepared from a mpBTL22 phage clone, and the DNA was subjected to in vitro mutagenesis using the oligonucleotide ZC1886

(Table 1) and the method described by Kunkel (Proc. Natl. Acad. Sci. USA 82: 488-492, 1985). A phage clone comprising the mutagenized PDGF β -R with a donor splice site (5' splice site) at the 3' end of the PDGF β -R extracellular domain was designated pBTLR-HX (Figure 7).

The native PDGF β -R signal sequence was obtained from pPR5. Plasmid pPR5, comprising 738 bp of 5' coding sequence with an Eco RI site immediately 5' to the translation initiation codon, was constructed by *in vitro* mutagenesis of the PDGF β -R cDNA fragment from RP51 (Example 1). Replicative form DNA of RP51 was digested with Eco RI to isolate the 1.09 kb PDGF β -R fragment. The PDGF β -R fragment was cloned into the Eco RI site of M13mp18. Single stranded template DNA was prepared from a phage clone containing the PDGF β -R fragment in the proper orientation. The template DNA was subjected to *in vitro* mutagenesis using oligonucleotide ZC1380 (Sequence ID Number 8; Table 1) and the method described by Zoller and Smith (Meth. Enzymol. 100: 468-500, 1983). The mutagenesis resulted in the placement of an Eco RI site immediately 5' to the translation initiation codon. Mutagenized phage clones were analyzed by dideoxy sequence analysis. A phage clone containing the ZC1380 mutation was selected, and replicative form (Rf) DNA was prepared from the phage clone. The Rf DNA was digested with Eco RI and Acc I to isolate the 0.63 kb fragment. Plasmid pR-RXI (Example 1) was digested with Acc I and Eco RI to isolate the 3.7 kb fragment. The 0.63 kb fragment and the 3.7 kb fragment were joined by ligation resulting in plasmid pPR5 (Figure 7).

As shown in Figure 7, the PDGF β -R signal peptide and part of the extracellular domain were obtained from plasmid pPR5 as a 1.4 kb Eco RI-Sph I fragment. Replicative form DNA from phage clone pBTLR-HX was digested with Sph I and Hind III to isolate the approximately 0.25 kb PDGF β -R fragment. Plasmid pUC19 was linearized by digestion with Eco RI and Hind III. The 1.4

kb Eco RI-Sph I PDGF β -R fragment, the 0.25 kb Sph I-Hind III fragment from pBTLR-HX and the Eco RI-Hind III cut pUC19 were joined in a three-part ligation. The resultant plasmid, pSDL110, was digested with Eco RI and Hind III to 5 isolate the 1.65 kb PDGF β -R fragment.

Plasmid pICHuC κ 3.9.11 was used as the source of the human immunoglobulin light chain gene (Figure 7). The human immunoglobulin light chain gene was isolated from a human genomic library using an oligonucleotide probe (5' 10 TGT GAC ACT CTC CTG GGA GTT A 3'; Sequence ID Number 32), which was based on a published human kappa C gene sequence (Hieter et al., Cell 22: 197-207, 1980). The human light chain (kappa) constant region was subcloned as a 1.1 kb Sph I-Hinf I genomic fragment of the human kappa gene, 15 which has been treated with DNA polymerase DNA I (Klenow Fragment) to fill in the Hinf I adhesive end, into Sph I-Hinc II cut pUC19. The 1.1 kb human kappa constant region was subsequently isolated as a 1.1 kb Sph I-Bam HI fragment that was subcloned into Sph I-Bgl II cut pIC19R 20 (Marsh et al., *ibid.*). The resultant plasmid was designated pICHuC κ 3.9.11. Plasmid pICHuC κ 3.9.11 was digested with Hind III and Eco RI to isolate the 1.1 kb kappa constant region gene. Plasmid pIC19H was linearized by digestion with Eco RI. The 1.65 kb PDGF β -R fragment, 25 the 1.1 kb human kappa constant region fragment and the linearized pIC19H were joined in a three part ligation. The resultant plasmid, pSDL112, was digested with Bam HI and Cla I to isolate the 2.75 kb fragment. Plasmid p μ PRE8 was linearized with Bgl II and Cla I. The 2.75 kb 30 fragment and the linearized p μ PRE8 were joined by ligation. The resultant plasmid was designated pSDL114 (Figure 7).

Plasmid pSDL114 was linearized by digestion with Cla I and was cotransfected with Pvu I-digested p416 into 35 SP2/0-Ag14 (ATCC CRL 1581) by electroporation using the method essentially described by Neumann et al. (EMBO J. 1: 841-845, 1982). (Plasmid p416 comprises the Adenovirus 5

ori, SV40 enhancer, Adenovirus 2 major late promoter, Adenovirus 2 tripartite leader, 5' and 3' splice sites, the DHFR^r cDNA, the SV40 polyadenylation signal and pML-1 (Lusky and Botchan, Nature 293: 79-81, 1981) vector sequences.) Transfectants were selected in growth medium containing methotrexate.

Media from drug resistant clones were tested for the presence of secreted PDGF β -receptor analogs by enzyme-linked immunosorbant assay (ELISA). Ninety-six well assay plates were prepared by incubating 100 μ l of 1 μ g/ml polyclonal goat anti-human kappa chain (Cappel Laboratories, Melvern, PA) diluted in phosphate buffered saline (PBS; Sigma) overnight at 4°C. Excess antibody was removed by three washes with 0.5% Tween 20 in PBS. One hundred microliters of spent media was added to each well, and the well were incubated for one hour at 4°C. Unbound proteins were removed by eight washes with 0.5% Tween 20 in PBS. One hundred microliters of peroxidase-conjugated goat anti-human kappa antibody (diluted 1:1000 in a solution containing 5% chicken serum (GIBCO-BRL) + 0.5% Tween 20 in PBS) was added to each well and the wells were incubated for one hour at 4°C. One hundred microliters of chromophore (100 μ l ABTS (2,2'-Azinobis(3-ethylbenzothiazoline sulfonic acid) diammonium salt; Sigma) + 1 μ l 30% H₂O₂ + 12.5 ml citrate/phosphate buffer (9.04 g/l citric acid, 10.16 g/l Na₂HPO₄)) was added to each well, and the wells were incubated to thirty minutes at room temperature. The samples were measured at 405 nm. The results of the assay showed that the PDGF β -R analog secreted by the transfectants contained an immunoglobulin light chain.

Spent media from drug resistant clones was also tested for the presence of secreted PDGF β -receptor analogs by immunoprecipitation. Approximately one million drug resistant transfectants were metabolically labeled by growth in DMEM medium lacking cysteine + 2% calf serum for 18 hours at 37°C in the presence of 50 μ CI ³⁵S-cysteine.

Media was harvested from the labeled cells and 250 μ l of the spent media was assayed by immunoprecipitation with the anti-PDGF β -receptor antibody PR7212 to detect the presence of metabolically labeled PDGF β -receptor analogs.

5 PR7212, diluted in PBS, was added to the media to a final concentration of 2.5 μ g per 250 μ l spent media. Five microliters of rabbit anti-mouse Ig diluted in PBS was added to the PR7212/media mixtures. The immunocomplexes were precipitated by the addition of 50 μ l 10% fixed Staph 10 A (weight/volume in PBS). The immunocomplexes were analyzed on 8% SDS-polyacrylamide gels followed by autoradiography overnight at -70°C. The results of the immunoprecipitation showed that the PDGF β -receptor analog secreted by the transfectants was bound by the anti-PDGF 15 β -receptor antibody. The combined results of the ELISA and immunoprecipitation assays showed that the PDGF β -receptor analog secreted by the transfectants contained both the PDGF β -receptor ligand-binding domain and the human light chain constant region.

20

C. Cotransfection of pSDL114 with an immunoglobulin heavy chain

Plasmid pSDL114 was cotransfected with p ϕ 5V_HhuC γ 1M-neo, which encodes a neomycin resistance gene expression unit and a complete mouse/human chimeric immunoglobulin heavy chain gene expression unit.

Plasmid p ϕ 5V_HhuC γ 1M-neo was constructed as follows. The mouse immunoglobulin heavy chain gene was isolated from a lambda genomic DNA library constructed 25 from the murine hybridoma cell line NR-ML-05 (Serafini et al., Eur. J. Nucl. Med. 14: 232, 1988) using an oligonucleotide probe designed to span the V_H/D/J_H junction (5' GCA TAG TAG TTA CCA TAT CCT CTT GCA CAG 3'; Sequence ID Number 33). The human immunoglobulin gamma-1 30 gene was isolated from a human genomic library using a cloned human gamma-4 constant region gene (Ellison et al., DNA 1: 11-18, 1981). The mouse immunoglobulin variable 35

region was isolated as a 5.3 kb Sst I-Hind III fragment from the original phage clone and the human gamma-1 C gene was obtained from the original phage clone as a 6.0 kb Hind III-Xho I fragment. The chimeric gamma-1 C gene was 5 created by joining the V_H and C_H fragments via the common Hind III site and incorporating them with the *E. coli* neomycin resistance gene expression unit into pIC19H to yield pφ5V_HhuC_γ1M-neo.

Plasmid pSDL114 was linearized by digestion with 10 Cla I and was co-transfected into SP2/O-Ag14 cells with Asp 718 linearized pφ5V_HhuC_γ1M-neo. The transfectants were selected in growth medium containing methotrexate and neomycin. Media from drug-resistant clones were tested for their ability to bind PDGF in a competition binding 15 assay.

The competition binding assay measured the amount of ¹²⁵I-PDGF left to bind to human dermal fibroblast cells after preincubation with the spent media from pSDL114-pφ5V_HhuC_γ1M-neo transfected cells. The media 20 were serially diluted in binding medium (Table 4). The dilutions were mixed with 0.5 ng of iodinated PDGF-BB or iodinated PDGF-AA, and the mixtures were incubated for two hours at room temperature. Three hundred micrograms of unlabeled PDGF-BB or unlabeled PDGF-AA was added to one 25 tube from each series. The sample mixtures were added to 24 well plates containing confluent human dermal fibroblast cells. The cells were incubated with the mixture for four hours at 4°C. The supernatants were aspirated from the wells, and the wells were rinsed three 30 times with phosphate buffered saline that was held a 4°C (PBS; Sigma, St. Louis, Mo.). Five hundred microliters of PBS + 1% NP-40 was added to each well, and the plates were shaken on a platform shaker for five minutes. The cells were harvested and the amount of iodinated PDGF was 35 determined. The results of the competition binding assay showed that the protein produced from pSDL114-pφ5V_HhuC_γ1M-

neo transfected cells was able to competitively bind PDGF-BB but did not bind PDGF-AA.

The PDGF β -receptor analog produced from a pSDL114-p ϕ 5V_HhuC γ 1M-neo transfectant was assayed to determine if the receptor analog was able to bind PDGF-BB with high affinity. Eight and one half milliliters of spent media containing the PDGF β -R analogs from a pSDL114-p ϕ 5V_HhuC γ 1M-neo transfectant was added to 425 μ l of Sepharose Cl-4B-Protein A beads (Sigma, St. Louis, MO), and the mixture was incubated for 10 minutes at 4°C. The beads were pelleted by centrifugation and washed with binding medium (Table 4). Following the wash the beads were resuspended in 8.5 ml of binding media, and 0.25 ml aliquots were dispensed to 1.5 ml tubes. Binding reactions were prepared by adding iodinated PDGF-BBTyr (Example 18.F.) diluted in DMEM + 10% fetal calf serum to the identical aliquots of receptor-bound beads to final PDGF-BBTyr concentrations of between 4.12 pM and 264 pM. Nonspecific binding was determined by adding a 100 fold excess of unlabeled BB to an identical set of binding reactions. Mixtures were incubated overnight at 4°C.

The beads were pelleted by centrifugation, and unbound PDGF-BB was removed with three washes in PBS. The beads were resuspended in 100 μ l of PBS and were counted. Results of the assay showed that the PDGF β -R analog was able to bind PDGF-BB with high affinity.

D. Construction of pSDL113.

As shown in Figure 8, the DNA sequence encoding the extracellular domain of the PDGF β -receptor was joined with the DNA sequence encoding a human immunoglobulin heavy chain constant region joined to a hinge sequence. Plasmid pSDL110 was digested with Eco RI and Hind III to isolate the 1.65 kb PDGF β -R fragment. Plasmid pICHu γ -1M was used as the source of the heavy chain constant region and hinge region. Plasmid pICHu γ -1M comprises the approximately 6 kb Hind III-Xho I fragment of a human

The mouse NR-ML-05 immunoglobulin light chain variable region gene was subcloned from the original mouse genomic phage clone into pIC19R as a 3 kb Xba I-Hinc II fragment. The human kappa C gene was subcloned from the original 5 human genomic phage clone into pUC19 as a 2.0 kb Hind III-Eco RI fragment. The chimeric kappa gene was created by joining the NR-ML-05 light chain variable region gene and human light chain constant region gene via the common Sph I site and incorporating them with the *E. coli* neomycin 10 resistance gene into pIC19H to yield pIC ϕ 5V κ HuC κ -Neo (Figure 9).

The linearized pSDL113 and pIC ϕ 5V κ HuC κ -Neo are transfected into SP2/0-Ag14 cells by electroporation. The transfectants are selected in growth medium containing 15 methotrexate and neomycin.

F. Cotransfection of pSDL113 and pSDL114

A clone of SP2/0-Ag14 stably transfected with pSDL114 and p416 was co-transfected with Cla I-digested 20 pSDL113 and Bam HI-digested pICneo by electroporation. (Plasmid pICneo comprises the SV40 promoter operatively linked to the *E. coli* neomycin resistance gene and pIC19H vector sequences.) Transfected cells were selected in growth medium containing methotrexate and G418. Media 25 from drug-resistant clones were tested for their ability to bind PDGF-BB or PDGF-AA in a competition binding assay as described in Example 12.C. The results of the assay showed that the transfectants secreted a PDGF β -receptor analog which was capable of competitively binding PDGF-BB 30 but did not detectably bind to PDGF-AA.

G. Cotransfection of pSDL114 with Fab

A clone of SP2/0-AG14 stably transfected with pSDL114 and p416 was transfected with the Fab region of 35 the human gamma-4 gene (γ_4) in plasmid p ϕ 5V H Fab-neo.

Plasmid p ϕ 5V H Fab-neo was constructed by first digesting plasmid p24BRH (Ellison et al., DNA 1: 11, 1988)

was digested with Xma I and Eco RI to isolate the 0.2 kb fragment comprising the immunoglobulin 3' untranslated region. Synthetic oligonucleotides ZC871 (Sequence ID Number 3; Table 1) and ZC872 (Sequence ID Number 4; Table 5 1) were kinased and annealed using essentially the methods described by Maniatis et al. (ibid.). The annealed oligonucleotides ZC871/ZC872 formed an Sst I-Xma I adapter. The ZC871/ZC872 adapter, the 0.2 kb p24BRH fragment and Sst I-Eco RI linearized pUC19 were joined in 10 a three-part ligation to form plasmid p γ 43'. Plasmid p γ 43' was linearized by digestion with Bam HI and Hind III. Plasmid p24BRH was cut with Hind III and Bgl II to isolate the 0.85 kb fragment comprising the C_H1 region. The p γ 43' fragment and the Hind III-Bgl II p24BRH fragment were 15 joined by ligation to form plasmid p γ 4Fab. Plasmid p γ 4Fab was digested with Hind III and Eco RI to isolate the 1.2 kb fragment comprising γ 4Fab. Plasmid pICneo, comprising the SV40 promoter operatively linked to the E. coli neomycin resistance gene and pIC19H vector sequences, was 20 linearized by digestion with Sst I and Eco RI. Plasmid p ϕ 5V_H, comprising the mouse immunoglobulin heavy chain gene variable region and pUC18 vector sequences, was digested with Sst I and Hind III to isolate the 5.3 kb V_H fragment. The linearized pICneo was joined with the 5.3 25 kb Sst I-Hind III fragment and the 1.2 kb Hind III-Eco RI fragment in a three-part ligation. The resultant plasmid was designated p ϕ 5V_HFab-neo (Figure 10).

A pSDL114/p416-transfected SP2/0-AG14 clone was transfected with Sca I-linearized p ϕ 5V_HFab-neo. 30 Transfected cells were selected in growth medium containing methotrexate and G418. Media from drug-resistant clones were tested for their ability to bind PDGF in a competition binding assay as described in Example 12.C. The results of the assay showed that the 35 PDGF β -receptor analog secreted from the transfectants was capable of competitively binding PDGF-BB.

H. Cotransfection of pSDL114 with Fab'

A stably transfected SP2/0-AG14 isolate containing pSDL114 and p416 was transfected with plasmid pWKI, which contained the Fab' portion of an 5 immunoglobulin heavy chain gene. Plasmid pWKI was constructed as follows.

The immunoglobulin gamma-1 Fab' sequence, comprising the C_H1 and hinge regions sequences, was derived from the gamma-1 gene clone described in Example 10 12.C. The gamma-1 gene clone was digested with Hind III and Eco RI to isolate the 3.0 kb fragment, which was — subcloned into Hind III-Eco RI linearized M13mp19. Single-stranded template DNA from the resultant phage was subjected to site-directed mutagenesis using 15 oligonucleotide ZC1447 (Sequence ID Number 9; Table 1) and essentially the method of Zoller and Smith (*ibid.*). A phage clone was identified having a ZC1447 induced deletion resulting in the fusion of the hinge region to a DNA sequence encoding the amino acids Ala-Leu-His-Asn-His- 20 Tyr-Thr-Glu-Ser-Leu-Ser-Leu-Ser-Pro-Gly-Lys (Sequence ID Number 31) followed in-frame by a stop codon. Replicative form DNA from a positive phage clone was digested with Hind III and Eco RI to isolate the 1.9 kb fragment comprising the C_H1 and hinge regions. Plasmid 25 pφ5V_H was digested with Sst I and Hind III to isolate the 5.3 kb fragment comprising the mouse immunoglobulin heavy chain gene variable region. Plasmid pICneo was linearized by digestion with Sst I and Eco RI. The linearized pICneo was joined with the 5.3 kb Hind III-Sst I fragment and the 30 1.9 kb Hind III-Eco RI fragment in a three-part ligation. The resultant plasmid was designated pWKI (Figure 10).

An SP2/0-AG14 clone stably transfected with pSDL114 and p416 was transfected with Asp 718-linearized pWKI. Transfected cells were selected by growth in medium 35 containing methotrexate and G418. Media samples from transfected cells were assayed using the competition assay described in Example 12.C. Results from the assays showed

that the transfected cells produced a PDGF β -receptor analog capable of competitively binding PDGF-BB.

Example 13

5 Purification and Characterization of PDGF β -Receptor
Analogs from Mammalian cells Co-transfected With pSDL113
and pSDL114

A. Purification of PDGF β -Receptor Analogs

10 The PDGF β -receptor analog was purified from conditioned culture media from a clone of transfected cells grown in a hollow fiber system. The media was passed over a protein-A sepharose column, and the column was washed sequentially with phosphate buffered saline, pH 15 7.2 (PBS; Sigma, St. Louis, MO) and 0.1 M citrate, pH 5.0. The PDGF β -receptor analog was eluted from the protein-A column with 0.1 M citrate pH 2.5 and immediately neutralized by the addition of Tris-base, pH 7.4. The eluate fractions containing PDGF β -receptor analog, as 20 determined by silver stain, were pooled and chromatographed over an S-200 column (Pharmacia LKB Technologies, Inc., Piscataway, NJ) equilibrated with PBS. The peak fractions from the S-200 column were pooled and concentrated on a centriprep-10 concentrator (Amicon). 25 Glycerol (10% final volume) was added to the preparation and the sample frozen at -80°C. PDGF β -receptor analogs purified from pSDL114 + pSDL113 co-transfected cells were termed "tetrameric PDGF α -receptors".

30 B. Measurement of The Relative Binding Affinity of Tetrameric PDGF β -Receptor Analog by Soluble Receptor Assay

Purified tetrameric PDGF β -receptor analog was compared to detergent solubilized extracts of human dermal 35 fibroblasts for 125 I-labeled PDGF-BB binding activity in a soluble receptor assay essentially as described by Hart et al. (J. Biol. Chem. 262: 10780-10785, 1987). Human dermal

fibroblast cells were extracted at 20×10^6 cell equivalents per ml in TNEN extraction buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 1 mM PMSF, 10% glycerol). Two hundred and fifty thousand PDGF β -receptor-subunits per cell was used to calculate the tetrameric PDGF β -receptor analog number per volume of extract. This value has been previously published by Seifert et al. (J. Biol Chem. 264: 8771-8778, 1989). The PDGF β -receptor analog number was determined from the protein concentration of the PDGF β -receptor analog assuming an average molecular weight of 140 kDa for each immunoglobulin-PDG β -receptor monomer, and four monomers per tetramer. Thus, each tetrameric molecule contains four receptor molecules.

Increasing amounts of either detergent solubilized extracts of human dermal fibroblast cells or purified PDGF β -receptor analog were incubated with 1ng of 125 I-labeled PDGF-BB for one hour at 37°C. The sample was then diluted with 1 ml binding media and was added to monolayers of human dermal fibroblast cells grown in 24-well culture dishes. The samples were incubated for two hours at 4°C. The wells were washed to remove unbound, 125 I-labeled PDGF-BB. On half of a milliliter of extraction buffer (PBS + 1% Nonidet P-40) was added to each well followed by a 5 minute incubation. The extraction mixtures were harvested and counted in a gamma counter.

The results showed that the PDGF β -receptor analog had the same relative binding affinity as solubilized PDGF β -receptor-subunit from mammalian cells in a solution phase binding assay.

C. Determination of the Binding Affinity of the PDGF β -Receptor Analog in a Solid Phase Format

The apparent dissociation constant $K_D(\text{app})$ of the PDGF β -receptor analog was determined essentially as described by Bowen-Pope and Ross (Methods in Enzymology

109: 69-100, 1985), using the concentration of ^{125}I -labeled PDGF-BB giving half-maximal specific ^{125}I -labeled PDGF-BB binding. Saturation binding assays to determine the concentration of ^{125}I -labeled PDGF-BB that gave half-maximal binding to immobilized PDGF β -receptor analog were conducted as follows.

Affinity purified goat anti-human IgG, H- and L-chain (Commercially available from Cappel Labs) was diluted into 0.1 M Na_2HCO_3 , pH 9.6 to a concentration of 2 $\mu\text{g}/\text{ml}$. One hundred microliters of the antibody solution was coated onto each well of 96-well microtiter plates for 18 hours at 4°C. The wells were washed once with ELISA C buffer (PBS + 0.05% Tween-20) followed by an incubation with 175 $\mu\text{l}/\text{well}$ of ELISA B buffer (PBS + 1% BSA + 0.05% Tween-20) to block the wells. The wells were washed once with ELISA B buffer. One hundred microliters of 12.1 ng/ml or 24.3 ng/ml of tetrameric PDGF β -receptor analog protein diluted in ELISA B was added to each well and the plates were incubated for 2 hours at 37°C. Unbound protein was removed from the wells by two washes with ELISA C. ^{125}I -labeled PDGF-BBTyr (Example 18.F.) was serially diluted into binding media (25 mM HEPES, pH 7.2, 0.25% rabbit serum albumin diluted in HAMs F-12 medium (GIBCO-BRL)), and 100 μl of the dilutions were added to the wells. The plates were incubated for two hours at room temperature. The unbound ^{125}I -labeled PDGF-BB was removed, and the wells were washed three times with binding media. Following the last wash, 100 μl of 0.1 M citrate, pH 2.5 was added to each well. After five minutes, the citrate buffer was removed, transferred to a tube and counted in a gamma counter. The counts reflect counts of ^{125}I -labeled PDGF-BBTyr bound by the receptor analog. Nonspecific binding for each concentration of ^{125}I -labeled PDGF-BBTyr was determined by a parallel assay wherein separate wells coated only with goat anti-human IgG were incubated with the ^{125}I -labeled PDGF-BB concentrations. Nonspecific binding was determined to be

2.8% of the total input counts per well and averaged 6% of the total counts bound.

Saturation binding assay on 12.1 and 24.3 ng/ml of tetrameric PDGF β -receptor analog gave half-maximal 5 binding at 0.8 and 0.82 ng/ml 125 I-labeled PDGF-BB_{Tyr}, respectively. By Scatchard analysis (Scatchard, Ann. NY Acad. Sci 51: 660-667, 1949) these values were shown to correspond to a K_D (app) of 2.7×10^{-11} which agree with the published values for PDGF receptors on mammalian 10 cells.

Example 14

Solid Phase Ligand Binding Assay Using the PDGF β -Receptor Analog

15 A. Solid Phase Radioreceptor Competition Binding Assay
In a solid phase radioreceptor competition binding assay (RRA), the wells of 96-well microtiter plates were coated with 100 μ l of 2 μ g/ml affinity purified goat anti-human IgG (Cappel Labs) diluted in 0.1 M 20 Na_2HCO_3 , pH 9.6. After an eighteen hour incubation at 4°C, the wells were washed once with ELISA C. The wells were blocked by incubation for 2 hours at 37°C with 175 μ l/well ELISA B. The wells were washed once with ELISA B then incubated for 2 hours at 37°C with 50 ng/ml 25 tetrameric PDGF β -receptor analog diluted in ELISA B. The unbound receptor was removed, and the test wells were incubated with increasing concentrations of serially diluted, unlabeled PDGF-BB (diluted in binding media. Following a two hour incubation at room temperature, the 30 wells were washed three times with binding media. One hundred microliters of 5 ng/ml 125 I-labeled PDGF-BB_{Tyr} (Example 18.F.) was added to each well, and the plates were incubated for an additional two hours at room 35 temperature. The wells were washed three times with binding media followed by a 5 minute incubation with 100 μ l/well of 0.1 M citrate, pH 2.5. The samples were harvested and counted in a gamma counter.

Radioreceptor assay (RRA) competition binding curves were generated for PDGF β -receptor analog protein plated at 48.6 ng/ml. The sensitivity of the assays is 1 ng/ml of PDGF-BB, with 8 ng/ml giving 50% inhibition in 5 125 I-PDGF-BB binding, and a working range between 1 and 32 ng/ml of PDGF-BB. The values were similar to those obtained using monolayers of SK-5 cells in an RRA.

B. Use of Tetrameric PDGF β -Receptor Analogs As
10 Antagonists for PDGF-Stimulated Mitogenesis.

A tetrameric PDGF β -receptor analog, purified as described in Example 13, was analyzed for the ability to neutralize PDGF-stimulated mitogenesis in mouse 3T3 cells. Increasing amounts of the purified tetrameric PDGF β -15 receptor analog were mixed with 5 ng of PDGF. The mixtures were then added to cultures of mouse 3T3 cells. The ability of the PDGF to stimulate a mitogenic response, as measured by the incorporation of 3 H-thymidine, was determined essentially as described (Raines and Ross,
20 Methods in Enzymology 109: 749-773, 1985, which is incorporated by reference herein). The tetrameric PDGF β -receptor analog demonstrated a dose response inhibition of PDGF-BB-stimulated 3 H-thymidine incorporation, while having essentially no effect on PDGF-AA- and PDGF-AB-
25 stimulated 3 H-thymidine incorporation.

C. Binding of Tetrameric PDGF β -receptor Analog to Immobilized PDGF.

A tetrameric PDGF β -receptor analog, purified as described in Example 13, was analyzed for its ability to bind to immobilized PDGF. PDGF-BB (100 ng/ml) was coated onto wells a 96-well microtiter plate, and the plates were incubated 18 hours at 4°C followed by one wash with ELISA C buffer. The wells were incubated for 2 hours 37°C with 30 ELISA B buffer to block the wells. Increasing concentrations of 125 I-labeled tetrameric PDGF β -receptor
35 analog, diluted in binding media, was added to the wells

for two hours at room temperature. The wells were washed four times with ELISA C buffer to remove unbound receptor analog. One hundred microliters of 1 M H₂SO₄ was added to each well and the plates were incubated for five minutes 5 at room temperature. The solution was then harvested and transferred to tubes to be counted in a gamma counter. Nonspecific binding was determined to be less than 10% of the total counts bound.

A receptor competition binding assay was 10 developed using this assay format. The assay was carried out as described above, and simultaneous to the addition of the ¹²⁵I-labeled tetrameric PDGF β -receptor analog, increasing amounts of PDGF-AA, AB or BB were added to the PDGF-BB coated wells. Under these conditions, only PDGF-BB 15 was found to significantly block the binding of the labeled PDGF β -receptor analog to the immobilized PDGF-BB.

Example 15

Construction and Expression of PDGFr-R Analogs in Cultured 20 Mouse Myeloma Cells

A. Construction of an optimized PDGFr-R cDNA

The PDGF α -receptor coding region was optimized for expression in mammalian cells as follows. The 5' end of the cDNA was modified to include an optimized Kozak 25 consensus translation initiation sequence (Kozak, Nuc. Acids Res. 12: 857-872, 1984) and Eco RI and Bam HI sites just 5' of the initiation methionine codon. Oligonucleotides ZC2181, ZC2182, ZC2183 and ZC2184 (Sequence ID Numbers 23, 24, 25 and 26, respectively; 30 Table 1) were designed to form, when annealed, an adapter having an Eco RI adhesive end, a Bam HI restriction site, a sequence encoding a Kozak consensus sequence 5' to the initiation methionine codon, a mammalian codon optimized sequence encoding amino acids 1-42 of Figure 11, and an 35 Eco RI adhesive end that destroys the Eco RI site within the PDGFr-R coding sequence. The adapter also introduced a diagnostic Cla I site 3' to the initiation methionine

codon. Oligonucleotides ZC2181, ZC2182, ZC2183 and ZC2184 were kinased, annealed and ligated. Plasmid p α 17B was linearized by partial digestion with Eco RI. The linearized p α 17B was ligated with the 5 ZC2181/ZC2182/ZC2183/ZC2184 oligonucleotide adapter, and the ligation mixture was transformed into *E. coli*. Plasmid DNA prepared from the transformants was analyzed by restriction analysis and a positive clone having the oligonucleotide adapter in the correct orientation was 10 digested with Eco RI and Pst I to isolate the 1.6 kb fragment. This fragment was subcloned into Eco RI + Pst I-linearized M13mp19. The resultant phage clone was designated 792-8. Single-stranded 792-8 DNA was sequenced to confirm the orientation of the adapter.

15 A fragment encoding the ligand-binding domain of the PDGF α -receptor (PDGFr-R) was then generated as follows. Restriction sites and a splice donor sequence were introduced at the 3' end of the PDGFr-R extracellular domain by PCR amplification of the 792-8 DNA and 20 oligonucleotides ZC2311 and ZC2392 (Sequence ID Numbers 27 and 30, Table 1). Oligonucleotide ZC2311 is a sense primer encoding nucleotides 1470 to 1489 of Figure 11. Oligonucleotide ZC2392 is an antisense primer that encodes 25 nucleotides 1759 to 1776 of Figure 11 followed by a splice donor and Xba I and Hind III restriction sites. The 792-8 DNA was amplified using manufacturer recommended (Perkin Elmer Cetus, Norwalk, CT) conditions and the GeneAmpTM DNA amplification reagent kit (Perkin Elmer Cetus), and blunted-ended 329 bp fragment was isolated. The blunt-end 30 fragment was digested with Nco I and Hind III and ligated with Sma I-digested pUC18. A plasmid having an insert with the Nco I site distal to the Hind III site present in the pUC18 polylinker was designated pUC18 Sma-PCR Nco HIII #13. The Hind III site present in the insert was not 35 regenerated upon ligation with the linearized pUC18. Plasmid pUC18 Sma-PCR Nco HIII #13 was digested with Nco I and Hind III to isolate the 355 bp PDGFr-R containing

fragment encoding PDGF α R. Oligonucleotides ZC2351 and ZC2352 (Table 1; Sequence ID Numbers 28 and 29) were kinased and annealed to form an Sst I-Nco I adapter encoding an internal Eco RI site and a Kozak consensus 5 translation initiation site. The 355 bp Nco I-Hind III fragment, the ZC2351/ZC2352 adapter and a 1273 bp Nco I fragment comprising the extracellular domain of of PDGF α -R derived from 792-8 were ligated with Hind III + SstI-digested pUC18 and tranformed into E. coli. Plasmid DNA 10 was isolated from the transformants and analyzed by restriction analysis. None of the isolates contained the 1273 bp Nco I fragment. A plasmid containing the Nco I-Hind III fragment and the ZC2351/ZC2352 adapter was desginated pUC18 Hin Sst Δ Nco #46. Plasmid pUC18 Hin Sst 15 Δ Nco #46 was linearized by digestion and joined by ligation with the 1273 bp Nco I fragment comprising the extracellular domain of the PDGF α -R from clone α 18 R-19. The ligations were transformed into E. coli, and plasmid DNA was isolated from the transformants. Analysis of the 20 plasmid DNA showed that only clones with the Nco I fragment in the wrong orientation were isolated. A clone having the Nco I fragment in the wrong orientation was digested with Nco I, religated and transformed into E. coli. Plasmid DNA was isolated from the transformants and 25 was analyzed by restriction analysis. A plasmid having the Nco I insert in the correct orientation was digested to completion with Hind III and partially digested with Sst I to isolate the 1.6 kb fragment comprising the extracellular domain of the PDGF α -R preceded by a 30 consensus initiation sequence (Kozak, ibid.) and followed by a splice donor site.

B. Construction of pPAB7

The DNA sequence encoding the extracellular 35 domain of the PDGF α -R was joined to the immunoglobulin μ enhancer-promoter and to a DNA sequence encoding an immunoglobulin light chain constant region. The

immunoglobulin μ enhancer-promoter was obtained from plasmid pJH1 which was derived from plasmid PIC μ PRES (Example 12.A.) by digestion with Eco RI and Sst I to isolate the 2.2 kb fragment comprising the immunoglobulin 5 enhancer and heavy chain variable region promoter. The 2.2 kb Sst I-Eco RI fragment was ligated with Sst I + Eco RI-linearized pUC19. The resulting plasmid, designated pJH1, contained the immunoglobulin enhancer and heavy chain variable region promoter immediately 5' to the pUC19 10 linker sequences. Plasmid pJH1 was linearized by digestion with Sst I and Hind III and joined with the 1.6 kb partial Sst I-Hind III fragment containing the PDGF α -R extracellular domain sequences. The resulting plasmid having the immunoglobulin μ enhancer-promoter joined to 15 the PDGF α -R extracellular domain was designated pPAB6. Plasmid pSDL112 was digested with Hind III to isolate the 1.2 kb fragment encoding the immunoglobulin light chain constant region (C_k). The 1.2 kb Hind III fragment was ligated with Hind III-linearized pPAB6. A plasmid having 20 the C_k sequence in the correct orientation was designated pPAB7.

C. Construction of pPAB9

The partial Sst I-Hind III fragment encoding the 25 extracellular domain of the PDGF α -R was joined to the immunoglobulin heavy chain constant region. For convenience, the internal Xba I site in plasmid pJH1 was removed by digestion with Xba I, blunt-ending with T4 DNA polymerase, and religation. A plasmid which did not 30 contain the internal Xba I site, but retained the Xba I site in the polylinker was designated 11.28.3.6. Plasmid 11.28.3.6 was linearized by digestion with Sst I and Xba I. Plasmid pPAB6 was digested to completion with Hind III and partially digested with Sst I to isolate the 1.6 kb 35 Sst I-Hind III fragment containing the PDGF α -R extracellular domain. Plasmid p ϕ 5V_HhuC γ 1M-neo (Example 12.C.) was digested wtih Hind III and Xba I to isolate the

6.0 kb fragment encoding the immunoglobulin heavy chain constant region (huC_γ1M). The Sst I-Hind III-linearized 11.28.3.6, the 1.6 kb Sst I-Hind III PDGF α -R fragment and the 6.0 kb Hind III-Xba I huC_γ1M fragment were ligated to 5 form plasmid pPAB9.

D. Expression of pPAB9 in Mammalian Cells

Bgl II-linearized pPAB7 and Pvu I-linearized pPAB9 were cotransfected with Pvu I-linearized p416 into 10 SP2/0-Ag14 cells by electroporation. Transfected cells were initially selected in growth medium containing 50 nM methotrexate and were subsequently amplified in a growth medium containing 100 μ M methotrexate. Media from drug resistant clones were tested for the presence of secreted 15 PDGF α -receptor analogs by enzyme-linked immunosorbant assay (ELISA). Ninety-six well assay plates were prepared by incubating 100 μ l of 1 μ g/ml monoclonal antibody 292.1.8 which is specific for the PDGF α -receptor diluted in phosphate buffered saline (PBS; Sigma) overnight at 4°C. 20 Excess antibody was removed by three washes with 0.5% Tween 20 in PBS. One hundred microliters of spent media was added to each well, and the plates were incubated for one hour at 4°C. Unbound proteins were removed by eight washes with 0.5% Tween 20 in PBS. One hundred microliters 25 of peroxidase-conjugated goat anti-human IgG heavy chain antibody (diluted 1:1000 in a solution containing 5% chicken serum (GIBCO-BRL) + 0.5% Tween 20 in PBS) was added to each well, and the plates were incubated for one hour at 4°C. One hundred microliters of chromophore (100 30 μ l ABTS [2,2'-Azinobis(3-ethylbenz-thiazoline sulfonic acid] diammonium salt; Sigma] + 1 μ l 30% H₂O₂ + 12.5 ml citrate/phosphate buffer [9.04 g/l citric acid, 10.16 g/l Na₂HPO₄]) was added to each well, and the wells were incubated for 30 minutes at room temperature. The samples 35 were measured at 405 nm. The results of the assay showed that the PDGF α -receptor analogs secreted by the transfectants contained an immunoglobulin heavy chain.

Analysis of spent media from transfected cells by Northern analysis, Western analysis and by radioimmunoprecipitation showed that the transfectants did not express a PDGF α -receptor analog from the pPAB7 5 construction. Transfectants were subsequently treated as containing only pPAB9.

Drug resistant clones was also tested for the presence of secreted PDGF α -receptor analogs by immunoprecipitation. For each clone, approximately one 10 million drug resistant transfectants were grown in DMEM lacking cysteine + 2% calf serum for 18 hours at 37°C in the presence of 50 μ Ci 35 S-cysteine. The spent media was harvested from the labeled cells and 250 μ l of medium from each clone was assayed for binding to the anti-PDGF α - 15 receptor antibody 292.18. Monoclonal antibody 292.18 diluted in PBS was added to each sample to a final concentration of 2.5 μ g per 250 μ l spent media. Five microliters of rabbit anti-mouse Ig diluted in PBS was added to each sample, and the immunocomplexes were 20 precipitated by the addition of 50 μ l 10% fixed Staph A (weight/volume in PBS). The immunocomplexes were analyzed on 8% SDS-polyacrylamide gels followed by autoradiography overnight at -70°C. The results of the immunoprecipitation showed that the PDGF α -receptor analog 25 secreted by the transfectants was bound by the anti-PDGF α -receptor antibody. The combined results of the ELISA and immunoprecipitation assays showed that the PDGF α -receptor analog secreted by the transfectants contained both the PDGF α -receptor ligand-binding domain and the 30 human heavy chain.

Spent medium from drug-resistant clones were tested for their ability to bind PDGF in a competition binding assay essentially as described in Example 12.C. The results of the assay showed that the transfectants 35 secreted a PDGF α -receptor analog capable of binding PDGF-AA. A clone containing the pPAB9 was designated 3.17.1.57.

E. Co-expression of pPAB7 and pPAB9 in Mammalian Cells

Bgl II-linearized pPAB7 and Bam HI-linearized pICneo were cotransfected into clone 3.17.1.57, and 5 transfected cells were selected in the presence of neomycin. Media from drug resistant cells were assayed for the presence of immunoglobulin heavy chain, immunoglobulin light chain and the PDGF α -receptor ligand-binding domain by ELISA essentially as described above.

10 Briefly, ninety-six well assay plates were prepared by incubating 100 μ l of 1 μ g/ml goat anti-human IgG Fc antibody (Sigma) or 100 μ l of 1 μ g/ml 292.18 overnight at 4°C. Excess antibody was removed by three washes with 0.5% Tween 20 in PBS. One hundred microliters of spent 15 media was added to each well of each plate, and the plates were incubated for one hour at 4°C. Unbound proteins were removed by eight washes with 0.5% Tween 20 in PBS. One hundred microliters of peroxidase-conjugated goat anti-human IgG antibody (diluted 1:1000 in a solution 20 containing 5% chicken serum (GIBCO-BRL) + 0.5% Tween 20 in PBS) was added to each well of the plate coated with the anti-Fc antibody, and 100 μ l of peroxidase-conjugated goat anti human kappa antibody (diluted 1:1000 in a solution containing 5% chicken serum (GIBCO-BRL) + 0.5% Tween 20 in 25 PBS) was added to each well of the plate coated with 292.18. The plates were incubated for one hour at 4°C. One hundred microliters of chromophore (100 μ l ABTS [2,2'-Azinobis(3-ethylbenz-thiazoline sulfonic acid) diammonium salt; Sigma] + 1 μ l 30% H₂O₂ + 12.5 ml citrate/phosphate 30 buffer [9.04 g/l citric acid, 10.16 g/l Na₂HPO₄]) was added to each well of each plate, and the plates were incubated to 30 minutes at room temperature. The samples were measured at 405 nm, the wavelength giving maximal absorbance of the chromogenic substrate, to identify 35 clones having absorbances higher than background indicating the presence of immunoglobulin heavy chain. Clones that gave positive results in both ELISA assays

(showing that the clones produced proteins containing heavy chain regions, light chain constant regions and the PDGF α -receptor ligand-binding region) were selected for further characterization.

- 5 Drug resistant clones that were positive for both ELISA assays were subsequently tested for the presence of secreted PDGF α -receptor analogs by immunoprecipitation. For each positive clone, approximately one million drug resistant transfectants
10 were grown in DMEM lacking cysteine + 2% calf serum for 18 hours at 37°C in the presence of 50 μ CI 35 S-cysteine. The spent media was harvested from the labeled cells and 250 μ l of medium from each clone was assayed for binding to monoclonal antibody 292.18. Monoclonal antibody 292.18
15 diluted in PBS was added to each sample to a final concentration of 2.5 μ g. Five microliters of rabbit anti-mouse Ig diluted in PBS was added to each sample and the immunocomplexes were precipitated by the addition of 50 μ l 10% fixed Staph A (weight/volume in PBS). The
20 immunocomplexes were analyzed on 8% SDS-polyacrylamide gels followed by autoradiography overnight at -70°C. The results of the immunoprecipitation showed that the PDGF α -receptor analog secreted by the transfectants was bound by the anti-PDGF α -receptor antibody. The combined results
25 of the ELISA and immunoprecipitation assays showed that the PDGF α -receptor analog secreted by the transfectants contained the PDGF α -receptor ligand-binding domain, the human heavy chain and the human light chain constant region. A clone that secreted a PDGF α -receptor analog
30 that was positive for both the above-described ELISA assays and the immunoprecipitation assay was designated 5.6.2.1.

Example 16Purification and Characterization of PDGF α -Receptor
Analogs5 A. Purification of PDGF α -Receptor Analogs From Clone
3.17.1.57

The PDGF α -Receptor analog was purified from the conditioned culture media of clone 3.17.1.57 by cycling cell-conditioned medium over an immunoaffinity column composed of monoclonal antibody 292.18 bound to a CNBr-activated Sepharose 4B resin, which is specific for the PDGF α -receptor. The column was washed with PBA, then eluted with 0.1 M citrate, pH 3.0. The peak column fractions containing the α -receptor were pooled, neutralized to pH 7.2 by the addition of 2 M Tris, pH 7.4, then passed over a protein-A Sepharose column. This column was washed sequentially with PBS, then with 0.1 M citrate, pH 5.0. The PDGF α -receptor analog was then eluted with 0.1 M citrate, pH 3.0. The peak eluate fractions were pooled, and glycerol was added to a final concentration of 10%. The sample was concentrated on a centriprep 10 concentrator (Amicon). The PDGF α -receptor analog purified from clone 3.17.1.57 was termed a "dimeric PDGF α -receptor analog".

25

B. Purification of PDGF α -Receptor Analogs From Clone
5.6.2.1

The PDGF α -receptor analog was purified from the conditioned culture media of clone 5.6.2.1 by cycling cell-conditioned medium over the immunoaffinity column described above. The column was washed with PBS then eluted with 0.1 M citrate, pH 3.0. The peak column fractions containing the α -receptor were pooled, neutralized to pH 7.2 by the addition of 2 M Tris (what pH 7.4), then passed over a protein-A sepharose column. This column was washed sequentially with PBS then with 0.1 M

citrate, pH 5.0. The PDGF α -receptor analog was then eluted with 0.1 M citrate, pH 3.0. The peak eluate fractions were pooled and glycerol was added to a final concentration of 10%. The sample was concentrated on a 5 centriprep 10 concentrator. The PDGF α -receptor analogs purified from clone 5.6.2.1 was termed a "tetrameric PDGF α -receptor analog".

Example 17

10 A. Use of the PDGF α -receptor Analogs in Ligand Binding Studies

Purified tetrameric PDGF α -receptor analog and purified dimeric PDGF α -receptor analog were compared to monolayers of a control cell line of canine kidney 15 epithelial cells, which do not naturally express the PDGF α -receptor, transfected with the human PDGF α -receptor cDNA for ligand binding activity. The dissociation constant (K_d) of the receptor preparations was determined by saturation binding and subsequent Scatchard analysis.

20 Ligand binding of the purified PDGF α -receptor analogs was determined using a solid phase binding assay. Affinity-purified goat anti-human IgG was diluted to a concentration of 2 μ g/ml in 0.1 M Na_2HCO_3 , pH 9.6 and 100 μ l/well of the solution was used to coat 96-well 25 microtiter plates for 18 hours at 4°C. Excess antibody was removed from the wells with one wash with ELISA C buffer (PBS, 0.05% Tween-20). The plates were incubated with 175 μ l/well of ELISA B buffer (PBS, 1% BSA, 0.05% Tween-20) to block the wells, followed by two washes with 30 ELISA C buffer. One hundred microliters of 50 ng/ml PDGF α -receptor analog (dimeric or tetrameric) diluted in ELISA buffer B was added to each well and the plates were incubated over night at 4°C. Unbound protein was removed from the wells with two washes with ELISA buffer B. ^{125}I -35 labeled PDGF-AA was serially diluted in binding media (Hams F-12, 25 mM HEPES pH 7.2, 0.25% rabbit serum albumin), and 100 μ l of each dilution was added to the

wells. The samples were incubated for two hours at room temperature. Unbound ^{125}I -labeled PDGF-AA was removed with three washes with binding media. One hundred microliters of 0.1 M citrate, pH 2.5 was added to each 5 well, and the plates were incubated for five minutes. After the incubation, the citrate buffer was removed and transferred to a tube for counting in a gamma counter. Nonspecific binding for each concentration of ^{125}I -labeled PDGF-AA was determined by a parallel assay wherein 10 separate wells coated only with goat anti-human IgG were incubated with the ^{125}I -labeled PDGF-AA samples.

— A saturation binding assay was performed on alpha T-7 cells transfected with the PDGF α -receptor. The cells were grown to confluence in 24-well culture plates. 15 The cells were washed one time with binding media. Iodinated PDGF-AA was serially diluted in binding media. One milliliter of each dilution was added to the wells, and the plates were incubated for 3 hours at 4°C . Unbound ^{125}I -labeled PDGF-AA was removed and the cells were washed 20 three times with binding media. PBS containing 1% Triton X-100 was added to the cells for 5 minutes. The extracts were harvested and counted in a gamma counter. Nonspecific binding was determined at a single concentration of ^{125}I -labeled PDGF-AA using a 500-fold 25 excess PDGF-BB.

The dissociation constants determined by Scatchard analysis (*ibid.*) of the saturation binding assays for the tetrameric PDGF α -receptor analog, dimeric PDGF α -receptor analog and the control cells (Table 5).

Table 5

Dissociation Constants for the Tetrameric PDGF α -Receptor,
the Dimeric PDGF α -receptor and control cells Transfected
with the PDGF α -receptor

5

<u>Receptor</u>	<u>KD</u>
Tetrameric PDGF α -receptor analog	- 1.6×10^{-11}
Dimeric PDGF α -receptor analog	- 8.51×10^{-11}
10 Control cells[PDGF α -receptor]	- 3.7×10^{-11}

A solid-phase competition binding assay was established using the tetrameric PDGF α -receptor analog. Ninety six-well microtiter plates were coated with goat 15 anti-human IgG (2 μ g/ml), the wells blocked with ELISA B buffer, 50 ng/ml of purified tetrameric PDGF α -receptor analog diluted in binding media was added, and the plates were incubated two hours at room temperature. Unbound receptor was removed and the wells were washed with 20 binding media. The plates were incubated for two hours at room temperature with increasing concentrations of either PDGF-AA or PDGF-BB diluted in binding media. The wells were washed, then incubated for two hours at room 25 temperature with 3 ng/ml 125 I-labeled PDGF-AA diluted in binding media. Unbound labeled PDGF-AA was removed, the wells were subsequently washed with binding media, and the bound, labeled PDGF-AA was harvested by the addition of 0.1 M citrate, pH 2.5, as described for the saturation 30 binding studies. PDGF-AB, PDGF-AA and PDGF-BB were found to compete for receptor binding with 125 I-PDGF-AA.

B. Use of Tetrameric PDGF α -Receptor Analogs As Antagonists for PDGF-Stimulated Mitogenesis.

A dimeric PDGF α -receptor analog, purified as 35 described in Example 16.B., was analyzed for the ability to neutralize PDGF-stimulated mitogenesis in mouse 3T3 cells. Increasing amounts of the purified tetrameric PDGF

α -receptor analog were mixed with PDGF-AA, -AB or -BB ranging .6 to 5 ng. The mixtures were then added to cultures of confluent mouse 3T3 cells. The ability of the PDGF to stimulate a mitogenic response, as measured by the incorporation of 3 H-thymidine, was determined essentially as described (Raines and Ross, Methods in Enzymology 109: 749-773, 1985, which is incorporated by reference herein). The dimeric PDGF α -receptor analog demonstrated a dose response inhibition of PDGF-stimulated 3 H-thymidine incorporation for all three isoforms of PDGF.

c. Inverse Ligand-Receptor Radioreceptor Assay
An inverse ligand-receptor radioreceptor assay was designed to screen for the presence of PDGF-BB, PDGF-BB binding proteins, PDGF-BB related molecules, and PDGF- β receptor antagonists in test samples. PDGF-BB (100 ng/ml) was coated onto the walls of 96-well microtiter plates, and the plates were incubated at 4°C for 16 hours. The wells were washed once with ELISA C buffer and then incubated with ELISA B buffer to block the nonspecific binding sites. To the wells were added 50 μ l of either PDGF standard or a test sample and 50 μ l of 125 I-labeled tetrameric PDGF β -receptor analog. The samples were incubated for one hour at room temperature. The wells were washed once with ELISA C buffer, and 0.1 M citrate, pH 2.5 containing 1% NP-40 was added to each well to disrupt the ligand-receptor analog bond and elute the bound receptor analog. The acid wash was collected and counted in a gamma counter. The presence of PDGF or a molecule which mimics PDGF or otherwise interferes with the binding of the well-bound PDGF-BB with its receptor will cause a decrease in the binding of the radiolabeled tetrameric PDGF β -receptor. Using this assay, PDGF-BB was found to inhibit receptor binding while PDGF-AA and PDGF-AB caused no significant decrease in receptor binding.

Example 18

Assay Methods

A. Preparation of Nitrocellulose Filters for Colony Assay

5 Colonies of transformants were tested for secretion of PDGF β -receptor analogs by first growing the cells on nitrocellulose filters that had been laid on top of solid growth medium. Nitrocellulose filters (Schleicher & Schuell, Keene, NH) were placed on top of
10 solid growth medium and were allowed to be completely wetted. Test colonies were patched onto the wetted filters and were grown at 30°C for approximately 40 hours. The filters were then removed from the solid medium, and the cells were removed by four successive rinses with
15 Western Transfer Buffer (Table 4). The nitrocellulose filters were soaked in Western Buffer A (Table 4) for one hour at room temperature on a shaking platform with two changes of buffer. Secreted PDGF β -R analogs were visualized on the filters described below.

20

B. Preparation of Protein Blot Filters

A nitrocellulose filter was soaked in Western Buffer A (Table 4) without the gelatin and placed in a Minifold (Schleicher & Schuell, Keene, NH). Five
25 milliliters of culture supernatant was added without dilution to the Minifold wells, and the liquid was allowed to pass through the nitrocellulose filter by gravity. The nitrocellulose filter was removed from the minifold and was soaked in Western Buffer A (Table 3) for one hour on a
30 shaking platform at room temperature. The buffer was changed three times during the hour incubation.

C. Preparation of Western Blot Filters

The transformants were analyzed by Western blot,
35 essentially as described by Towbin et al. (Proc. Natl. Acad. Sci. USA 76: 4350-4354, 1979) and Gordon et al. (U.S. Patent No. 4,452,901). Culture supernatants from

- appropriately grown transformants were diluted with three volumes of 95% ethanol. The ethanol mixtures were incubated overnight at -70°C. The precipitates were spun out of solution by centrifugation in an SS-24 rotor at 5 18,000 rpm for 20 minutes. The supernatants were discarded and the precipitate pellets were resuspended in 200 μ l of dH₂O. Two hundred microliters of 2x loading buffer (Table 4) was added to each sample, and the samples were incubated in a boiling water bath for 5 minutes.
- 10 The samples were electrophoresed in a 15% sodium dodecylsulfate polyacrylamide gel under non-reducing conditions. The proteins were electrophoretically transferred to nitrocellulose paper using conditions described by Towbin et al. (*ibid.*). The nitrocellulose 15 filters were then incubated in Western Buffer A (Table 4) for 75 minutes at room temperature on a platform rocker.

D. Processing the Filters for Visualization with Antibody

- 20 Filters prepared as described above were screened for proteins recognized by the binding of a PDGF β -receptor specific monoclonal antibody, designated PR7212. The filters were removed from the Western Buffer A (Table 4) and placed in sealed plastic bags containing a 25 10 ml solution comprising 10 μ g/ml PR7212 monoclonal antibody diluted in Western Buffer A. The filters were incubated on a rocking platform overnight at 4°C or for one hour at room temperature. Excess antibody was removed with three 15-minute washes with Western Buffer A on a 30 shaking platform at room temperature.
- Ten microliters biotin-conjugated horse anti-mouse antibody (Vector Laboratories, Burlingame, CA) in 20 ml Western Buffer A was added to the filters. The filters were re-incubated for one hour at room temperature on a 35 platform shaker, and unbound conjugated antibody was removed with three fifteen-minute washes with Western Buffer A.

- The filters were pre-incubated for one hour at room temperature with a solution comprising 50 μ l Vectastain Reagent A (Vector Laboratories) in 10 ml of Western Buffer A that had been allowed to incubate at room
- 5 temperature for 30 minutes before use. The filters were washed with one quick wash with distilled water followed by three 15-minute washes with Western Buffer B (Table 4) at room temperature. The Western Buffer B washes were followed by one wash with distilled water.
- 10 During the preceding wash step, the substrate reagent was prepared. Sixty mg of horseradish peroxidase reagent (Bio-Rad, Richmond, CA) was dissolved in 20 ml HPLC grade methanol. Ninety milliliters of distilled water was added to the dissolved peroxidase followed by
- 15 2.5 ml 2 M Tris, pH 7.4 and 3.8 ml 4 M NaCl. One hundred microliters of 30% H_2O_2 was added just before use. The washed filters were incubated with 75 ml of substrate and incubated at room temperature for 10 minutes with vigorous shaking. After the 10 minute incubation, the buffer was
- 20 changed, and the filters were incubated for an additional 10 minutes. The filters were then washed in distilled water for one hour at room temperature. Positives were scored as those samples which exhibited coloration.
- 25 E. Processing the Filters For Visualization with an Anti-Substance P Antibody
- Filters prepared as described above were probed with an anti-substance P antibody. The filters were removed from the Western Buffer A and rinsed with Western
- 30 transfer buffer, followed by a 5-minute wash in phosphate buffered saline (PBS, Sigma, St. Louis, MO). The filters were incubated with a 10 ml solution containing 0.5 M 1-ethyl-3-3-dimethylamino propyl carbodiimide (Sigma) in 1.0 M NH_4Cl for 40 minutes at room temperature. After
- 35 incubation, the filters were washed three times, for 5 minutes per wash, in PBS. The filters were blocked with 2% powdered milk diluted in PBS.

The filters were then incubated with a rat anti-substance P monoclonal antibody (Accurate Chemical & Scientific Corp., Westbury, NY). Ten microliters of the antibody was diluted in 10 ml of antibody solution (PBS 5 containing 20% fetal calf serum and 0.5% Tween-20). The filters were incubated at room temperature for 1 hour. Unbound antibody was removed with four 5-minute washes with PBS.

The filters were then incubated with a biotin-10 conjugated rabbit anti-rat peroxidase antibody (Cappel Laboratories, Melvern, PA). The conjugated antibody was diluted 1:1000 in 10 ml of antibody solution for 2 hours at room temperature. Excess conjugated antibody was removed with four 5-minute washes with PBS.

15 The filters were pre-incubated for 30 minutes at room temperature with a solution containing 50 μ l Vectastain Reagent A (Vector Laboratories) and 50 μ l Vectastain Reagent B (Vector Laboratories) in 10 ml of antibody solution that had been allowed to incubate for 30 20 minutes before use. Excess Vectastain reagents were removed by four 5-minute washes with PBS.

During the preceding wash step, the substrate reagent was prepared. Sixty milligrams of horseradish peroxidase reagent (Bio-Rad Laboratories, Richmond, CA) 25 was dissolved in 25 ml of HPLC grade methanol. Approximately 100 ml of PBS and 200 μ l H_2O_2 were added just before use. The filters were incubated with the substrate reagent for 10 to 20 minutes. The substrate was removed by a vigorous washing distilled water.

30

F. Iodination of PDGF-BB.

A PDGF-BB mutant molecule having a tyrosine replacing the phenylalanine at position 23 (PDGF-BB_{Tyr}) was iodinated and subsequently purified, using a purification 35 method which produces ¹²⁵I-labeled PDGF-BB with a higher specific activity than primary-labeled material and which was found to substantially decrease the nonspecific binding

component. The PDGF-BB_{Tyr} was labeled using the Iodobead method (Pierce Chemical). The labeled protein was gel filtered over a C-25 desalting column (Pharmacia LKB Technologies) equilibrated with 10 mM acetic acid, 0.25% 5 gelatin and 100 mM NaCl. The peak fractions were pooled and pH adjusted to 7.2 by the addition of Tris-base. The labeled mixture was chromatographed over an affinity column composed of PDGF β -receptor analog protein coupled to CnBr-activated Sepharose (Pharmacia LKB Technologies, 10 Inc.). The column was washed with phosphate buffered saline and eluted with 0.1 M citrate, pH 2.5 containing 0.25% gelatin. The peak eluate fractions were pooled and assayed by ELISA to determine the PDGF-BB concentration.

Although the foregoing invention has been 15 described in some detail by way of illustration and example for purposes of clarity of understanding, it will be evident that certain changes and modifications may be practiced within the scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Sledziewski Ph.D., Andrzej Z
Bell, Lillian A.
Kindsvogel Ph.D., Wayne R.

(ii) TITLE OF INVENTION: METHODS OF PRODUCING SECRETED RECEPTOR ANALOGS
AND BIOLOGICALLY ACTIVE DIMERIZED POLYPEPTIDE
FUSIONS

(iii) NUMBER OF SEQUENCES: 36

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Seed and Berry
(B) STREET: 6300 Columbia Center
(C) CITY: Seattle
(D) STATE: WA
(E) COUNTRY: USA
(F) ZIP: 98104-7092

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.24

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(B) FILING DATE:
(C) CLASSIFICATION:

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(ix) ATTORNEY/AGENT INFORMATION:

(A) NAME: Maki J.D., David J.
(B) REGISTRATION NUMBER: 31,392
(C) REFERENCE/DOCKET NUMBER: 990008.446C3

(x) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 206-622-4900
(B) TELEFAX: 206-682-6031
(C) TELEX: 3723836

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 4465 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Homo sapiens
- (D) DEVELOPMENTAL STAGE: Adult
- (F) TISSUE TYPE: Skin
- (G) CELL TYPE: fibroblasts

(vii) IMMEDIATE SOURCE:

- (B) CLONE: pR-rX1

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 354..3671
- (D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CCCTCAGCCC TGCTGCCAG CACGAGCCTG TGCTGCCCT GCCCCACGCA GACAGCCAGA	60
CCCAGGGCGG CCCCTCTGGC GGCTCTGCTC CTCCCGAAGG ATGCTTGGGG AGTGAGGCAG	120
AGCTGGGCAG TCCTCTCCCC TACAGCAGCC CCCTTCCTCC ATCCCTCTGT TCTCCTGAGC	180
CTTCAGGAGC CTGCAACCAGT CCTGCCTGTC CTTCTACTCA GCTGTTACCC ACTCTGGGAC	240
CAGCAGTCTT TCTGATAACT GGGAGAGGGC AGTAAGGAGG ACTTCCTGGA GGGGGTGACT	300
GTCCAGAGCC TGGAACTGTG CCCACACCAAG AAGCCATCAG CAGCAAGGAC ACC ATG Met 1	356
CGG CTT CCG GGT GCG ATG CCA GCT CTG GCC CTC AAA GGC GAG CTG CTG Arg Leu Pro Gly Ala Met Pro Ala Leu Ala Leu Lys Gly Glu Leu Leu	404
5 10 15	
TTG CTG TCT CTC CTG TTA CTT CTG GAA CCA CAG ATC TCT CAG GGC CTG Leu Leu Ser Leu Leu Leu Leu Glu Pro Gln Ile Ser Gln Gly Leu	452
20 25 30	

GTC GTC ACA CCC CCG GGG CCA GAG CTT GTC CTC AAT GTC TCC AGC ACC Val Val Thr Pro Pro Gly Pro Glu Leu Val Leu Asn Val Ser Ser Thr 35 40 45	500
TTC GTT CTG ACC TGC TCG GGT TCA GCT CCG GTG GTG TGG GAA CGG ATG Phe Val Leu Thr Cys Ser Gly Ser Ala Pro Val Val Trp Glu Arg Met 50 55 60 65	548
TCC CAG GAG CCC CCA CAG GAA ATG GCC AAG GCC CAG GAT GGC ACC TTC Ser Gln Glu Pro Pro Gln Glu Met Ala Lys Ala Gln Asp Gly Thr Phe 70 75 80	596
TCC AGC GTG CTC ACA CTG ACC AAC CTC ACT GGG CTA GAC ACG GGA GAA Ser Ser Val Leu Thr Leu Thr Asn Leu Thr Gly Leu Asp Thr Gly Glu 85 90 95	644
TAG TTT TGC ACC CAC AAT GAC TCC CGT GGA CTG GAG ACC GAT GAG CGG Tyr Phe Cys Thr His Asn Asp Ser Arg Gly Leu Glu Thr Asp Glu Arg 100 105 110	692
AAA CGG CTC TAC ATC TTT GTG CCA GAT CCC ACC GTG GGC TTC CTC CCT Lys Arg Leu Tyr Ile Phe Val Pro Asp Pro Thr Val Gly Phe Leu Pro 115 120 125	740
AAT GAT GCC GAG GAA CTA TTC ATC TTT CTC ACG GAA ATA ACT GAG ATC Asn Asp Ala Glu Glu Leu Phe Ile Phe Leu Thr Glu Ile Thr Glu Ile 130 135 140 145	788
ACC ATT CCA TGC CGA GTA ACA GAC CCA CAG CTG GTG GTG ACA CTG CAC Thr Ile Pro Cys Arg Val Thr Asp Pro Gln Leu Val Val Thr Leu His 150 155 160	836
GAG AAG AAA GGG GAC GTT GCA CTG CCT GTC CCC TAT GAT CAC CAA CGT Glu Lys Lys Gly Asp Val Ala Leu Pro Val Pro Tyr Asp His Gln Arg 165 170 175	884
GGC TTT TCT GGT ATC TTT GAG GAC AGA AGC TAC ATC TGC AAA ACC ACC Gly Phe Ser Gly Ile Phe Glu Asp Arg Ser Tyr Ile Cys Lys Thr Thr 180 185 190	932
ATT GGG GAC AGG GAG GTG GAT TCT GAT GCC TAC TAT GTC TAC AGA CTC Ile Gly Asp Arg Glu Val Asp Ser Asp Ala Tyr Tyr Val Tyr Arg Leu 195 200 205	980
CAG GTG TCA TCC ATC AAC GTC TCT GTG AAC GCA GTG CAG ACT GTG GTC Gln Val Ser Ser Ile Asn Val Ser Val Asn Ala Val Gln Thr Val Val 210 215 220 225	1028
CGC CAG GGT GAG AAC ATC ACC CTC ATG TGC ATT GTG ATC GGG AAT GAG Arg Gln Gly Glu Asn Ile Thr Leu Met Cys Ile Val Ile Gly Asn Glu 230 235 240	1076
GTG GTC AAC TTC GAG TGG ACA TAC CCC CGC AAA GAA AGT GGG CGG CTG Val Val Asn Phe Glu Trp Thr Tyr Pro Arg Lys Glu Ser Gly Arg Leu 245 250 255	1124

GTG GAG CCG GTG ACT GAC TTC CTC TTG GAT ATG CCT TAC CAC ATC CGC Val Glu Pro Val Thr Asp Phe Leu Leu Asp Met Pro Tyr His Ile Arg 260 265 270	1172
TCC ATC CTG CAC ATC CCC AGT GCC GAG TTA GAA GAC TCG GGG ACC TAC Ser Ile Leu His Ile Pro Ser Ala Glu Leu Glu Asp Ser Gly Thr Tyr 275 280 285	1220
ACC TGC AAT GTG ACG GAG AGT GTG AAT GAC CAT CAG GAT GAA AAG GCC Thr Cys Asn Val Thr Glu Ser Val Asn Asp His Gln Asp Glu Lys Ala 290 295 300 305	1268
ATC AAC ATC ACC GTG GTT GAG AGC GGC TAC GTG CGG CTC CTG GGA GAG Ile Asn Ile Thr Val Val Glu Ser Gly Tyr Val Arg Leu Leu Gly Glu 310 315 320	1316
<u>GTG</u> GGC ACA CTA CAA TTT GCT GAG CTG CAT CGG AGC CGG ACA CTG CAG Val Gly Thr Leu Gln Phe Ala Glu Leu His Arg Ser Arg Thr Leu Gln 325 330 335	1364
GTA GTG TTC GAG GCC TAC CCA CCG CCC ACT GTC CTG TGG TTC AAA GAC Val Val Phe Glu Ala Tyr Pro Pro Pro Thr Val Leu Trp Phe Lys Asp 340 345 350	1412
AAC CGC ACC CTG GGC GAC TCC AGC GCT GGC GAA ATC GCC CTG TCC ACG Asn Arg Thr Leu Gly Asp Ser Ser Ala Gly Glu Ile Ala Leu Ser Thr 355 360 365	1460
CGC AAC GTG TCG GAG ACC CCG TAT GTG TCA GAG CTG ACA CTG GTT CGC Arg Asn Val Ser Glu Thr Arg Tyr Val Ser Glu Leu Thr Leu Val Arg 370 375 380 385	1508
GTG AAG GTG GCA GAG GCT GGC CAC TAC ACC ATG CGG GCC TTC CAT GAG Val Lys Val Ala Glu Ala Gly His Tyr Thr Met Arg Ala Phe His Glu 390 395 400	1556
GAT GCT GAG GTC CAG CTC TCC TTC CAG CTA CAG ATC AAT GTC CCT GTC Asp Ala Glu Val Gln Leu Ser Phe Gln Leu Gln Ile Asn Val Pro Val 405 410 415	1604
CGA GTG CTG GAG CTA AGT GAG AGC CAC CCT GAC AGT GGG GAA CAG ACA Arg Val Leu Glu Leu Ser Glu Ser His Pro Asp Ser Gly Glu Gln Thr 420 425 430	1652
GTC CGC TGT CGT GGC CGG GGC ATG CCC CAG CCG AAC ATC ATC TGG TCT Val Arg Cys Arg Gly Arg Gly Met Pro Gln Pro Asn Ile Ile Trp Ser 435 440 445	1700
GCC TGC AGA GAC CTC AAA AGG TGT CCA CGT GAG CTG CCG CCC ACG CTG Ala Cys Arg Asp Leu Lys Arg Cys Pro Arg Glu Leu Pro Pro Thr Leu 450 455 460 465	1748

CTG GGG AAC AGT TCC GAA GAG GAG AGC CAG CTG GAG ACT AAC GTG ACG Leu Gly Asn Ser Ser Glu Glu Glu Ser Gln Leu Glu Thr Asn Val Thr 470 475 480	1796
TAC TGG GAG GAG GAG CAG GAG TTT GAG GTG GTG AGC ACA CTG CGT CTG Tyr Trp Glu Glu Glu Gln Glu Phe Glu Val Val Ser Thr Leu Arg Leu 485 490 495	1844
CAG CAC GTG GAT CGG CCA CTG TCG GTG CGC TGC ACG CTG CGC AAC GCT Gln His Val Asp Arg Pro Leu Ser Val Arg Cys Thr Leu Arg Asn Ala 500 505 510	1892
G TG GGC CAG GAC ACG CAG GAG GTC ATC GTG GTG CCA CAC TCC TTG CCC Val Gly Gln Asp Thr Gln Glu Val Ile Val Val Pro His Ser Leu Pro 515 520 525	1940
TTT AAG GTG GTG GTG ATC TCA GCC ATC CTG GCC CTG GTG GTG CTC ACC Phe Lys Val Val Val Ile Ser Ala Ile Leu Ala Leu Val Val Leu Thr 530 535 540 545	1988
ATC ATC TCC CTT ATC ATC CTC ATC ATG CTT TGG CAG AAG AAG CCA CGT Ile Ile Ser Leu Ile Ile Leu Ile Met Leu Trp Gln Lys Lys Pro Arg 550 555 560	2036
TAC GAG ATC CGA TGG AAG GTG ATT GAG TCT GTG AGC TCT GAC GGC CAT Tyr Glu Ile Arg Trp Lys Val Ile Glu Ser Val Ser Ser Asp Gly His 565 570 575	2084
GAG TAC ATC TAC GTG GAC CCC ATG CAG CTG CCC TAT GAC TCC ACG TGG Glu Tyr Ile Tyr Val Asp Pro Met Gln Leu Pro Tyr Asp Ser Thr Trp 580 585 590	2132
GAG CTG CCG CGG GAC CAG CTT GTG CTG GGA CGC ACC CTC GGC TCT GGG Glu Leu Pro Arg Asp Gln Leu Val Leu Gly Arg Thr Leu Gly Ser Gly 595 600 605	2180
GCC TTT GGG CAG GTG GTG GAG GCC ACG GCT CAT GGC CTG AGC CAT TCT Ala Phe Gly Gln Val Val Glu Ala Thr Ala His Gly Leu Ser His Ser 610 615 620 625	2228
CAG GCC ACG ATG AAA GTG GCC GTC AAG ATG CTT AAA TCC ACA GCC CGC Gln Ala Thr Met Lys Val Ala Val Lys Met Leu Lys Ser Thr Ala Arg 630 635 640	2276
AGC AGT GAG AAG CAA GCC CTT ATG TCG GAG CTG AAG ATC ATG AGT CAC Ser Ser Glu Gln Ala Leu Met Ser Glu Leu Lys Ile Met Ser His 645 650 655	2324
CTT GGG CCC CAC CTG AAC GTG GTC AAC CTG TTG GGG GCC TGC ACC AAA Leu Gly Pro His Leu Asn Val Val Asn Leu Leu Gly Ala Cys Thr Lys 660 665 670	2372
GGA GGA CCC ATC TAT ATC ATC ACT GAG TAC TGC CGC TAC GGA GAC CTG Gly Gly Pro Ile Tyr Ile Ile Thr Glu Tyr Cys Arg Tyr Gly Asp Leu 675 680 685	2420

GTG GAC TAC CTG CAC CGC AAC AAA CAC ACC TTC CTG CAG CAC CAC TCC Val Asp Tyr Leu His Arg Asn Lys His Thr Phe Leu Gln His His Ser 690 695 700 705	2468
GAC AAG CGC CGC CCC AGC GCG GAG CTC TAC AGC AAT GCT CTG CCC Asp Lys Arg Arg Pro Pro Ser Ala Glu Leu Tyr Ser Asn Ala Leu Pro 710 715 720	2516
GTT GGG CTC CCC CTG CCC AGC CAT GTG TCC TTG ACC GGG GAG AGC GAC Val Gly Leu Pro Leu Pro Ser His Val Ser Leu Thr Gly Glu Ser Asp 725 730 735	2564
GGT GGC TAC ATG GAC ATG AGC AAG GAC GAG TCG GTG GAC TAT GTG CCC Gly Gly Tyr Met Asp Met Ser Lys Asp Glu Ser Val Asp Tyr Val Pro 740 745 750	2612
ATG CTG GAC ATG AAA GGA GAC GTC AAA TAT GCA GAC ATC GAG TCC TCC Met Leu Asp Met Lys Gly Asp Val Lys Tyr Ala Asp Ile Glu Ser Ser 755 760 765	2660
AAC TAC ATG GCC CCT TAC GAT AAC TAC GTT CCC TCT GCC CCT GAG AGG Asn Tyr Met Ala Pro Tyr Asp Asn Tyr Val Pro Ser Ala Pro Glu Arg 770 775 780 785	2708
ACC TGC CGA GCA ACT TTG ATC AAC GAG TCT CCA GTG CTA AGC TAC ATG Thr Cys Arg Ala Thr Leu Ile Asn Glu Ser Pro Val Leu Ser Tyr Met 790 795 800	2756
GAC CTC GTG GGC TTC AGC TAC CAG GTG GCC AAT GGC ATG GAG TTT CTG Asp Leu Val Gly Phe Ser Tyr Gln Val Ala Asn Gly Met Glu Phe Leu 805 810 815	2804
GCC TCC AAG AAC TGC GTC CAC AGA GAC CTG GCG GCT AGG AAC GTG CTC Ala Ser Lys Asn Cys Val His Arg Asp Leu Ala Ala Arg Asn Val Leu 820 825 830	2852
ATC TGT GAA GGC AAG CTG GTC AAG ATC TGT GAC TTT GGC CTG GCT CGA Ile Cys Glu Gly Lys Leu Val Lys Ile Cys Asp Phe Gly Leu Ala Arg 835 840 845	2900
GAC ATC ATG CGG GAC TCG AAT TAC ATC TCC AAA GGC AGC ACC TTT TTG Asp Ile Met Arg Asp Ser Asn Tyr Ile Ser Lys Gly Ser Thr Phe Leu 850 855 860 865	2948
CCT TTA AAG TGG ATG GCT CCG GAG AGC ATC TTC AAC AGC CTC TAC ACC Pro Leu Lys Trp Met Ala Pro Glu Ser Ile Phe Asn Ser Leu Tyr Thr 870 875 880	2996
ACC CTG AGC GAC GTG TGG TCC TTC GGG ATC CTG CTC TGG GAG ATC TTC Thr Leu Ser Asp Val Trp Ser Phe Gly Ile Leu Leu Trp Glu Ile Phe 885 890 895	3044

ACC TTG GGT GGC ACC CCT TAC CCA GAG CTG CCC ATG AAC GAG CAG TTC Thr Leu Gly Gly Thr Pro Tyr Pro Glu Leu Pro Met Asn Glu Gln Phe 900 905 910	3092
TAC AAT GCC ATC AAA CGG GGT TAC CGC ATG GCC CAG CCT GCC CAT GCC Tyr Asn Ala Ile Lys Arg Gly Tyr Arg Met Ala Gln Pro Ala His Ala 915 920 925	3140
TCC GAC GAG ATC TAT GAG ATC ATG CAG AAG TGC TGG GAA GAG AAG TTT Ser Asp Glu Ile Tyr Glu Ile Met Gln Lys Cys Trp Glu Glu Lys Phe 930 935 940 945	3188
GAG ATT CGG CCC TTC TCC CAG CTG GTG CTG CTT CTC GAG AGA CTG Glu Ile Arg Pro Pro Phe Ser Gln Leu Val Leu Leu Glu Arg Leu 950 955 960	3236
TTG GGC GAA GGT TAC AAA AAG AAG TAC CAG CAG GTG GAT GAG GAG TTT Leu Gly Glu Gly Tyr Lys Lys Tyr Gln Gln Val Asp Glu Glu Phe 965 970 975	3284
CTG AGG AGT GAC CAC CCA GCC ATC CTT CGG TCC CAG GCC CGC TTG CCT Leu Arg Ser Asp His Pro Ala Ile Leu Arg Ser Gln Ala Arg Leu Pro 980 985 990	3332
GGG TTC CAT GGC CTC CGA TCT CCC CTG GAC ACC AGC TCC GTC CTC TAT Gly Phe His Gly Leu Arg Ser Pro Leu Asp Thr Ser Ser Val Leu Tyr 995 1000 1005	3380
ACT GCC GTG CAG CCC AAT GAG GGT GAC AAC GAC TAT ATC ATC CCC CTG Thr Ala Val Gln Pro Asn Glu Gly Asp Asn Asp Tyr Ile Ile Pro Leu 1010 1015 1020 1025	3428
CCT GAC CCC AAA CCC GAG GTT GCT GAC GAG GGC CCA CTG GAG GGT TCC Pro Asp Pro Lys Pro Glu Val Ala Asp Glu Gly Pro Leu Glu Gly Ser 1030 1035 1040	3476
CCC AGC CTA GCC AGC TCC ACC CTG AAT GAA GTC AAC ACC TCC TCA ACC Pro Ser Leu Ala Ser Ser Thr Leu Asn Glu Val Asn Thr Ser Ser Thr 1045 1050 1055	3524
ATC TCC TGT GAC AGC CCC CTG GAG CCC CAG GAC GAA CCA GAG CCA GAG Ile Ser Cys Asp Ser Pro Leu Glu Pro Gln Asp Glu Pro Glu Pro Glu 1060 1065 1070	3572
CCC CAG CTT GAG CTC CAG GTG GAG CCG GAG CCA GAG CTG GAA CAG TTG Pro Gln Leu Glu Leu Gln Val Glu Pro Glu Pro Glu Leu Glu Gln Leu 1075 1080 1085	3620
CCG GAT TCG GGG TGC CCT GCG CCT CGG GCG GAA GCA GAG GAT AGC TTC Pro Asp Ser Gly Cys Pro Ala Pro Arg Ala Glu Ala Glu Asp Ser Phe 1090 1095 1100 1105	3668
CTG TAGGGGGCTG GCCCTACCC TGCCCTGCCT GAAGCTCCCC CCCTGCCAGC Leu	3721

ACCCAGCATC TCCTGGCCTG GCCTGACCGG GCTTCCTGTC AGCCAGGCTG CCCTTATCAG	3781
CTGTCCCCCTT CTGGAAGCTT TCTGCTCCTG ACGTGTTGTG CCCCCAACCC TGGGGCTGGC	3841
TTAGGAGGCA AGAAAAGTC ACCAGGCCGTG ACCAGCCCTC TGCCCTCCAGG GAGGCCAACT	3901
GACTCTGAGC CAGGGTTCCC CCAGGGAACT CAGTTTCCC ATATGTAAGA TGGGAAAGTT	3961
AGGCTTGATG ACCCAGAACATC TAGGATTCTC TCCCTGGCTG ACACGGTGGG GAGACCGAAT	4021
CCCTCCCTGG GAAGATTCTT GGAGTTACTG AGGTGGTAAA TTAACATTT TTCTGTTTCAG	4081
CCAGCTACCC CTCAAGGAAT CATAGCTCTC TCCTCGCACT TTTTATCCAC CCAGGAGCTA	4141
GGGAAGAGAC CCTAGCCTCC CTGGCTGCTG GCTGAGCTAG GGCCTAGCTT GAGCAGTGT	4201
GCCTCATCCA GAAGAAAGCC AGTCTCCTCC CTATGATGCC AGTCCCTGCG TTCCCTGGCC	4261
CGAGCTGGTC TGGGCCATT AGGCAGCCTA ATTAATGCTG GAGGCTGAGC CAAGTACAGG	4321
ACACCCCCAG CCTGCAGCCC TTGCCCAGGG CACTTGGAGC ACACGCAGCC ATAGCAAGTG	4381
CCTGTGTCCC TGTCCCTCAG GCCCATCAGT CCTGGGGCTT TTTCTTATC ACCCTCAGTC	4441
TTAATCCATC CACCAGAGTC TAGA	4465

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 1106 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Arg Leu Pro Gly Ala Met Pro Ala Leu Ala Leu Lys Gly Glu Leu			
1	5	10	15
Leu Leu Leu Ser Leu Leu Leu Leu Glu Pro Gln Ile Ser Gln Gly			
20	25	30	
Leu Val Val Thr Pro Pro Gly Pro Glu Leu Val Leu Asn Val Ser Ser			
35	40	45	
Thr Phe Val Leu Thr Cys Ser Gly Ser Ala Pro Val Val Trp Glu Arg			
50	55	60	
Met Ser Gln Glu Pro Pro Gln Glu Met Ala Lys Ala Gln Asp Gly Thr			
65	70	75	80

Phe Ser Ser Val Leu Thr Leu Thr Asn Leu Thr Gly Leu Asp Thr Gly
 85 90 95
 Glu Tyr Phe Cys Thr His Asn Asp Ser Arg Gly Leu Glu Thr Asp Glu
 100 105 110
 Arg Lys Arg Leu Tyr Ile Phe Val Pro Asp Pro Thr Val Gly Phe Leu
 115 120 125
 Pro Asn Asp Ala Glu Glu Leu Phe Ile Phe Leu Thr Glu Ile Thr Glu
 130 135 140
 Ile Thr Ile Pro Cys Arg Val Thr Asp Pro Gln Leu Val Val Thr Leu
 145 150 155 160
 His Glu Lys Lys Gly Asp Val Ala Leu Pro Val Pro Tyr Asp His Gln
 165 170 175
 Arg Gly Phe Ser Gly Ile Phe Glu Asp Arg Ser Tyr Ile Cys Lys Thr
 180 185 190
 Thr Ile Gly Asp Arg Glu Val Asp Ser Asp Ala Tyr Tyr Val Tyr Arg
 195 200 205
 Leu Gln Val Ser Ser Ile Asn Val Ser Val Asn Ala Val Gln Thr Val
 210 215 220
 Val Arg Gln Gly Glu Asn Ile Thr Leu Met Cys Ile Val Ile Gly Asn
 225 230 235 240
 Glu Val Val Asn Phe Glu Trp Thr Tyr Pro Arg Lys Glu Ser Gly Arg
 245 250 255
 Leu Val Glu Pro Val Thr Asp Phe Leu Leu Asp Met Pro Tyr His Ile
 260 265 270
 Arg Ser Ile Leu His Ile Pro Ser Ala Glu Leu Glu Asp Ser Gly Thr
 275 280 285
 Tyr Thr Cys Asn Val Thr Glu Ser Val Asn Asp His Gln Asp Glu Lys
 290 295 300
 Ala Ile Asn Ile Thr Val Val Glu Ser Gly Tyr Val Arg Leu Leu Gly
 305 310 315 320
 Glu Val Gly Thr Leu Gln Phe Ala Glu Leu His Arg Ser Arg Thr Leu
 325 330 335
 Gln Val Val Phe Glu Ala Tyr Pro Pro Pro Thr Val Leu Trp Phe Lys
 340 345 350
 Asp Asn Arg Thr Leu Gly Asp Ser Ser Ala Gly Glu Ile Ala Leu Ser
 355 360 365

Thr Arg Asn Val Ser Glu Thr Arg Tyr Val Ser Glu Leu Thr Leu Val
 370 375 380
 Arg Val Lys Val Ala Glu Ala Gly His Tyr Thr Met Arg Ala Phe His
 385 390 395 400
 Glu Asp Ala Glu Val Gln Leu Ser Phe Gln Leu Gln Ile Asn Val Pro
 405 410 415
 Val Arg Val Leu Glu Leu Ser Glu Ser His Pro Asp Ser Gly Glu Gln
 420 425 430
 Thr Val Arg Cys Arg Gly Arg Gly Met Pro Gln Pro Asn Ile Ile Trp
 435 440 445
 Ser Ala Cys Arg Asp Leu Lys Arg Cys Pro Arg Glu Leu Pro Pro Thr
 450 455 460
 Leu Leu Gly Asn Ser Ser Glu Glu Glu Ser Gln Leu Glu Thr Asn Val
 465 470 475 480
 Thr Tyr Trp Glu Glu Gln Glu Phe Glu Val Val Ser Thr Leu Arg
 485 490 495
 Leu Gln His Val Asp Arg Pro Leu Ser Val Arg Cys Thr Leu Arg Asn
 500 505 510
 Ala Val Gly Gln Asp Thr Gln Glu Val Ile Val Val Pro His Ser Leu
 515 520 525
 Pro Phe Lys Val Val Val Ile Ser Ala Ile Leu Ala Leu Val Val Leu
 530 535 540
 Thr Ile Ile Ser Leu Ile Ile Leu Ile Met Leu Trp Gln Lys Lys Pro
 545 550 555 560
 Arg Tyr Glu Ile Arg Trp Lys Val Ile Glu Ser Val Ser Ser Asp Gly
 565 570 575
 His Glu Tyr Ile Tyr Val Asp Pro Met Gln Leu Pro Tyr Asp Ser Thr
 580 585 590
 Trp Glu Leu Pro Arg Asp Gln Leu Val Leu Gly Arg Thr Leu Gly Ser
 595 600 605
 Gly Ala Phe Gly Gln Val Val Glu Ala Thr Ala His Gly Leu Ser His
 610 615 620
 Ser Gln Ala Thr Met Lys Val Ala Val Lys Met Leu Lys Ser Thr Ala
 625 630 635 640
 Arg Ser Ser Glu Lys Gln Ala Leu Met Ser Glu Leu Lys Ile Met Ser
 645 650 655

His Leu Gly Pro His Leu Asn Val Val Asn Leu Leu Gly Ala Cys Thr
 660 665 670
 Lys Gly Gly Pro Ile Tyr Ile Ile Thr Glu Tyr Cys Arg Tyr Gly Asp
 675 680 685
 Leu Val Asp Tyr Leu His Arg Asn Lys His Thr Phe Leu Gln His His
 690 695 700
 Ser Asp Lys Arg Arg Pro Pro Ser Ala Glu Leu Tyr Ser Asn Ala Leu
 705 710 715 720
 Pro Val Gly Leu Pro Leu Pro Ser His Val Ser Leu Thr Gly Glu Ser
 725 730 735
 Asp Gly Gly Tyr Met Asp Met Ser Lys Asp Glu Ser Val Asp Tyr Val
 740 745 750
 Pro Met Leu Asp Met Lys Gly Asp Val Lys Tyr Ala Asp Ile Glu Ser
 755 760 765
 Ser Asn Tyr Met Ala Pro Tyr Asp Asn Tyr Val Pro Ser Ala Pro Glu
 770 775 780
 Arg Thr Cys Arg Ala Thr Leu Ile Asn Glu Ser Pro Val Leu Ser Tyr
 785 790 795 800
 Met Asp Leu Val Gly Phe Ser Tyr Gln Val Ala Asn Gly Met Glu Phe
 805 810 815
 Leu Ala Ser Lys Asn Cys Val His Arg Asp Leu Ala Ala Arg Asn Val
 820 825 830
 Leu Ile Cys Glu Gly Lys Leu Val Lys Ile Cys Asp Phe Gly Leu Ala
 835 840 845
 Arg Asp Ile Met Arg Asp Ser Asn Tyr Ile Ser Lys Gly Ser Thr Phe
 850 855 860
 Leu Pro Leu Lys Trp Met Ala Pro Glu Ser Ile Phe Asn Ser Leu Tyr
 865 870 875 880
 Thr Thr Leu Ser Asp Val Trp Ser Phe Gly Ile Leu Leu Trp Glu Ile
 885 890 895
 Phe Thr Leu Gly Gly Thr Pro Tyr Pro Glu Leu Pro Met Asn Glu Gln
 900 905 910
 Phe Tyr Asn Ala Ile Lys Arg Gly Tyr Arg Met Ala Gln Pro Ala His
 915 920 925
 Ala Ser Asp Glu Ile Tyr Glu Ile Met Gln Lys Cys Trp Glu Glu Lys
 930 935 940

Phe Glu Ile Arg Pro Pro Phe Ser Gln Leu Val Leu Leu Leu Glu Arg
 945 950 955 960
 Leu Leu Gly Glu Gly Tyr Lys Lys Lys Tyr Gln Gln Val Asp Glu Glu
 965 970 975
 Phe Leu Arg Ser Asp His Pro Ala Ile Leu Arg Ser Gln Ala Arg Leu
 980 985 990
 Pro Gly Phe His Gly Leu Arg Ser Pro Leu Asp Thr Ser Ser Val Leu
 995 1000 1005
 Tyr Thr Ala Val Gln Pro Asn Glu Gly Asp Asn Asp Tyr Ile Ile Pro
 1010 1015 1020
 Leu Pro Asp Pro Lys Pro Glu Val Ala Asp Glu Gly Pro Leu Glu Gly
 1025 1030 1035 1040
 Ser Pro Ser Leu Ala Ser Ser Thr Leu Asn Glu Val Asn Thr Ser Ser
 1045 1050 1055
 Thr Ile Ser Cys Asp Ser Pro Leu Glu Pro Gln Asp Glu Pro Glu Pro
 1060 1065 1070
 Glu Pro Gln Leu Glu Leu Gln Val Glu Pro Glu Pro Glu Leu Glu Gln
 1075 1080 1085
 Leu Pro Asp Ser Gly Cys Pro Ala Pro Arg Ala Glu Ala Glu Asp Ser
 1090 1095 1100
 Phe Leu
 1105

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 57 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vii) IMMEDIATE SOURCE:

- (B) CLONE: ZC871

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATTATACGCT CTCTTCCTCA GGTAAATGAG TGCCAGGGCC GGCAAGCCCC CGCTCCA

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 56 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vii) IMMEDIATE SOURCE:

(B) CLONE: ZC872

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CCGGGGAGCG GGGGCTGCC GGCCCTGGCA CTCATTTACC TGAGGAAGAG AGAGCT

56

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vii) IMMEDIATE SOURCE:

(B) CLONE: ZC904

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CATGGGCACG TAATCTATAG ATTCAATCCTT GCTCATATCC ATGTA

45

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: Other nucleic acid
- (iii) HYPOTHETICAL: N
- (iv) ANTI-SENSE: N
- (vii) IMMEDIATE SOURCE:
 - (B) CLONE: ZC906

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

AAGCTGTCCT CTGCTTCAGC CAGAGGTCTT GGGCAGCC

38

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 38 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

- (vii) IMMEDIATE SOURCE:
 - (B) CLONE: ZC906

(xii) SEQUENCE DESCRIPTION: SEQ ID NO:7:

AAGCTGTCCT CTGCTTCAGC CAGAGGTCTT GGGCAGCC

38

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

- (vii) IMMEDIATE SOURCE:
 - (B) CLONE: ZC1380

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CATGGTGAA TTCTGCTGA T

21

(2) INFORMATION FOR SEQ ID NO:9:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 29 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vii) IMMEDIATE SOURCE:

- (B) CLONE: ZC1447

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

TGGTTTGCA GAGCTGAGGA AGAGATGGA

29

(2) INFORMATION FOR SEQ ID NO:10:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 55 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vii) IMMEDIATE SOURCE:

- (B) CLONE: ZC1453

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

AATTCAATTAT GTTGTGCAA GCCTTCTTGT TCCTGCTAGC TGGTTTCGCT GTTAA

55

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 55 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC1454

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GATCTTAAACA GCGAAACCAAG CTAGCAGGAA CAAGAAGGCT TGCAACAACA TAATG

55

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC1478

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

ATCGCGAGCA TGCAGATCTG A

21

(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

- (iii) HYPOTHETICAL: N
- (iv) ANTI-SENSE: N
- (vii) IMMEDIATE SOURCE:
 - (B) CLONE: ZC1479

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

AGCTTCAGAT CTGCATGCTG CCGAT

25

(2) INFORMATION FOR SEQ ID NO:14:

- (1) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 52 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

- (vii) IMMEDIATE SOURCE:
 - (B) CLONE: ZC1776

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

AGCTGACCGC AAATGTTGTG TCGAGTGCCC ACCGTGCCA GCTTAGAATT CT

52

(2) INFORMATION FOR SEQ ID NO:15:

- (1) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 52 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

- (vii) IMMEDIATE SOURCE:
 - (B) CLONE: ZC1777

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CTAGAGAATT CTAAGCTGGG CACGGTGGGC ACTCGACACA ACATTTGCGC TC 52

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 95 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vii) IMMEDIATE SOURCE:

(B) CLONE: ZC1846

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GATCGGCCAC TGTCGGTGGC CTGCACGCTG CGCAACGCTG TGGGCCAGGA CACGCAGGAG 60

GTCATCGTGG TGCCACACTC CTTGCCCTTT AAGCA 95

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 95 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vii) IMMEDIATE SOURCE:

(B) CLONE: ZC1847

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

AGCTTGCTTA AAGGGCAAGG AGTGTGGCAC CACGATGACC TCCTGCGTGT CCTGGCCCAC 60

AGCGTTGGCG AGCGTGACAG GCACCGACAG TGGCC 95

(2) INFORMATION FOR SEQ ID NO:18:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 43 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC1886

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CCAGTGCCAA GCTTGTCTAG ACTTACCTTT AAAGGGCAAG GAG

43

(2) INFORMATION FOR SEQ ID NO:19:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC1892

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

AGCTTGAGCG T

11

(2) INFORMATION FOR SEQ ID NO:20:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

- (iii) HYPOTHETICAL: N
- (iv) ANTI-SENSE: N
- (vii) IMMEDIATE SOURCE:
 - (B) CLONE: ZC1893

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

CTAGACGCTC A

11

(2) INFORMATION FOR SEQ ID NO:21:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 47 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

- (vii) IMMEDIATE SOURCE:
 - (B) CLONE: ZC1894

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

AGCTTCCAGT TCTTCGGCCT CATGTCAGTT CTTCGGCCTC ATGTGAT

47

(2) INFORMATION FOR SEQ ID NO:22:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 47 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

- (vii) IMMEDIATE SOURCE:
 - (B) CLONE: ZC1895

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

CTAGATCACA TGAGGCCGAA GAACTGACAT GAGGCCGAAG AACTGGA

47

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 66 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vii) IMMEDIATE SOURCE:

- (B) CLONE: ZC2181

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

AATTCCGGATC CACCATGGGC ACCAGCCACC CGGCCTTCCT GGTGTTAGGC TGCCTGCTGA

60

CCGGCC

66

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 71 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vii) IMMEDIATE SOURCE:

- (B) CLONE: ZC2182

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

TGAGCCTGAT CCTGTGCCAA CTGAGCCTGC CATCGATCCT GCCAACGAG AACGAGAAGG

60

TTGTGCAGCT A

71

(2) INFORMATION FOR SEQ ID NO:25:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 69 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vii) IMMEDIATE SOURCE:
 (B) CLONE: ZC2183

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

AATTTAGCTG CACAACCTTC TCGTTCTCGT TTGGCAGGAT CGATGGCAGG CTCAGTTGGC	60
ACAGGATCA	69

(2) INFORMATION FOR SEQ ID NO:26:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 68 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vii) IMMEDIATE SOURCE:
 (B) CLONE: ZC2184

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

GGCTCAGGCC GGTCAGCAGG CAGCCTAACCA CCAGGAACGC CGGGTGGCTG GTGCCCATGG	60
TGGATCCG	68

(2) INFORMATION FOR SEQ ID NO:27:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20 base pairs
 (B) TYPE: nucleic acid

- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC2311

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

TGATCACCAT GGCTCAACTG

20

(2) INFORMATION FOR SEQ ID NO:28:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 10 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC2351

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:28:

CGAATTCCAC

10

(2) INFORMATION FOR SEQ ID NO:29:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vii) IMMEDIATE SOURCE:
 (B) CLONE: ZC2352

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:
 ATTATACGCA TGGTGGATT CGAGCT 26

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 41 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vii) IMMEDIATE SOURCE:
 (B) CLONE: ZC2392

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:
 ACGTAAGCTT GTCTAGACTT ACCTTCAGAA CGCAGGGTGG G 41

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 17 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: C-terminal

(vii) IMMEDIATE SOURCE:
 (B) CLONE: pWK1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

Ala Leu His Asn His Tyr Thr Glu Lys Ser Leu Ser Leu Ser Pro Gly
 1 5 10 15

Lys

(2) INFORMATION FOR SEQ ID NO:32:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

TGTGACACTC TCCTGGGAGT TA

22

(2) INFORMATION FOR SEQ ID NO:33:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:33:

GCATAGTAGT TACCATATCC TCTTGCACAG

30

(2) INFORMATION FOR SEQ ID NO:34:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

ACCGAACGTG AGAGGGAGTGC TATAA 25

(2) INFORMATION FOR SEQ ID NO:35:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 4054 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(vii) IMMEDIATE SOURCE:

(B) CLONE: p-alpha-17B

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 205..3471
- (D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

GCCCTGGGGA CGGACCGTGG GCGGCGCGCA GCGGGCGGAC GCGTTTTGGG GACGTTGGTGG 60

CCAGCGCCTT CCTGCAGACC CACAGGAAG TACTCCCTT GACCTCCGGG GAGCTCCGAC 120

CAGGTTATAC GTTGCTGGTG GAAAAGTGAC AATTCTAGGA AAAGAGCTAA AAGCCGGATC 180

GGTGACCGAA AGTTTCCCAG AGCT ATG GGG ACT TCC CAT CCG GCG TTC CTG 231
Met Gly Thr Ser His Pro Ala Phe Leu
1 5GTC TTA GGC TGT CTT CTC ACA GGG CTG AGC CTA ATC CTC TGC CAG CTT 279
Val Leu Gly Cys Leu Leu Thr Gly Leu Ser Leu Ile Leu Cys Gln Leu
10 15 20 25TCA TTA CCC TCT ATC CTT CCA AAT GAA AAT GAA AAG GTT GTG CAG CTG 327
Ser Leu Pro Ser Ile Leu Pro Asn Glu Asn Glu Lys Val Val Gln Leu
30 35 40AAT TCA TCC TTT TCT CTG AGA TGC TTT GGG GAG AGT GAA GTG AGC TGG 375
Asn Ser Ser Phe Ser Leu Arg Cys Phe Gly Glu Ser Glu Val Ser Trp
45 50 55

CAG TAC CCC ATG TCT GAA GAA GAG AGC TCC GAT GTG GAA ATC AGA AAT Gln Tyr Pro Met Ser Glu Glu Glu Ser Ser Asp Val Glu Ile Arg Asn 60 65 70	423
GAA GAA AAC AAC AGC GGC CTT TTT GTG ACG GTC TTG GAA GTG AGC AGT Glu Glu Asn Asn Ser Gly Leu Phe Val Thr Val Leu Glu Val Ser Ser 75 80 85	471
GCC TCG GCG GCC CAC ACA GGG TTG TAC ACT TGC TAT TAC AAC CAC ACT Ala Ser Ala Ala His Thr Gly Leu Tyr Thr Cys Tyr Tyr Asn His Thr 90 95 100 105	519
CAG ACA GAA GAG AAT GAG CTT GAA GGC AGG CAC ATT TAC ATC TAT GTG Gln Thr Glu Glu Asn Glu Leu Glu Gly Arg His Ile Tyr Ile Tyr Val 110 115 120	567
CCA GAC CCA GAT GTA GCC TTT GTA CCT CTA GGA ATG ACG GAT TAT TTA Pro Asp Pro Asp Val Ala Phe Val Pro Leu Gly Met Thr Asp Tyr Leu 125 130 135	615
GTC ATC GTG GAG GAT GAT GAT TCT GCC ATT ATA CCT TGT CGC ACA ACT Val Ile Val Glu Asp Asp Asp Ser Ala Ile Ile Pro Cys Arg Thr Thr 140 145 150	663
GAT CCC GAG ACT CCT GTA ACC TTA CAC AAC AGT GAG GGG GTG GTA CCT Asp Pro Glu Thr Pro Val Thr Leu His Asn Ser Glu Gly Val Val Pro 155 160 165	711
GCC TCC TAC GAC AGC AGA CAG GGC TTT AAT GGG ACC TTC ACT GTA GGG Ala Ser Tyr Asp Ser Arg Gln Gly Phe Asn Gly Thr Phe Thr Val Gly 170 175 180 185	759
CCC TAT ATC TGT GAG GCC ACC GTC AAA GGA AAG AAG TTC CAG ACC ATC Pro Tyr Ile Cys Glu Ala Thr Val Lys Gly Lys Lys Phe Gln Thr Ile 190 195 200	807
CCA TTT AAT GTT TAT GCT TTA AAA GCA ACA TCA GAG CTG GAT CTA GAA Pro Phe Asn Val Tyr Ala Leu Lys Ala Thr Ser Glu Leu Asp Leu Glu 205 210 215	855
ATG GAA GCT CTT AAA ACC GTG TAT AAG TCA GGG GAA ACG ATT GTG GTC Met Glu Ala Leu Lys Thr Val Tyr Lys Ser Gly Glu Thr Ile Val Val 220 225 230	903
ACC TGT GCT GTT TTT AAC AAT GAG GTG GTT GAC CTT CAA TGG ACT TAC Thr Cys Ala Val Phe Asn Asn Glu Val Val Asp Leu Gln Trp Thr Tyr 235 240 245	951
CCT GGA GAA GTG AAA GGC AAA GGC ATC ACA ATA CTG GAA GAA ATC AAA Pro Gly Glu Val Lys Gly Lys Gly Ile Thr Ile Leu Glu Glu Ile Lys 250 255 260 265	999
GTC CCA TCC ATC AAA TTG GTG TAC ACT TTG ACG GTC CCC GAG GCC ACG Val Pro Ser Ile Lys Leu Val Tyr Thr Leu Thr Val Pro Glu Ala Thr 270 275 280	1047

GTG AAA GAC AGT GGA GAT TAC GAA TGT GCT GCC CGC CAG GCT ACC AGG Val Lys Asp Ser Gly Asp Tyr Glu Cys Ala Ala Arg Gln Ala Thr Arg 285 290 295	1095
GAG GTC AAA GAA ATG AAG AAA GTC ACT ATT TCT GTC CAT GAG AAA GGT Glu Val Lys Glu Met Lys Lys Val Thr Ile Ser Val His Glu Lys Gly 300 305 310	1143
TTC ATT GAA ATC AAA CCC ACC TTC AGC CAG TTG GAA GCT GTC AAC CTG Phe Ile Glu Ile Lys Pro Thr Phe Ser Gln Leu Glu Ala Val Asn Leu 315 320 325	1191
CAT GAA GTC AAA CAT TTT GTT GTA GAG GTG CGG GCC TAC CCA CCT CCC His Glu Val Lys His Phe Val Val Glu Val Arg Ala Tyr Pro Pro Pro 330 335 340 345	1239
AGG ATA TCC TGG CTG AAA AAC AAT CTG ACT CTG ATT GAA AAT CTC ACT Arg Ile Ser Trp Leu Lys Asn Asn Leu Thr Leu Ile Glu Asn Leu Thr 350 355 360	1287
GAG ATC ACC ACT GAT GTG GAA AAG ATT CAG GAA ATA AGG TAT CGA AGC Glu Ile Thr Thr Asp Val Glu Lys Ile Gln Glu Ile Arg Tyr Arg Ser 365 370 375	1335
AAA TTA AAG CTG ATC CGT GCT AAG GAA GAC AGT GGC CAT TAT ACT Lys Leu Lys Leu Ile Arg Ala Lys Glu Glu Asp Ser Gly His Tyr Thr 380 385 390	1383
ATT GTA GCT CAA AAT GAA GAT GCT GTG AAG AGC TAT ACT TTT GAA CTG Ile Val Ala Gln Asn Glu Asp Ala Val Lys Ser Tyr Thr Phe Glu Leu 395 400 405	1431
TTA ACT CAA GTT CCT TCA TCC ATT CTG GAC TTG GTC GAT GAT CAC CAT Leu Thr Gln Val Pro Ser Ser Ile Leu Asp Leu Val Asp Asp His His 410 415 420 425	1479
GGC TCA ACT GGG GGA CAG ACG GTG AGG TGC ACA GCT GAA GGC ACG CCG Gly Ser Thr Gly Gly Gln Thr Val Arg Cys Thr Ala Glu Gly Thr Pro 430 435 440	1527
CTT CCT GAT ATT GAG TGG ATG ATA TGC AAA GAT ATT AAG AAA TGT AAT Leu Pro Asp Ile Glu Trp Met Ile Cys Lys Asp Ile Lys Lys Cys Asn 445 450 455	1575
AAT GAA ACT TCC TGG ACT ATT TTG GCC AAC AAT GTC TCA AAC ATC ATC Asn Glu Thr Ser Trp Thr Ile Leu Ala Asn Asn Val Ser Asn Ile Ile 460 465 470	1623
ACG GAG ATC CAC TCC CGA GAC AGG AGT ACC GTG GAG GGC CGT GTG ACT Thr Glu Ile His Ser Arg Asp Arg Ser Thr Val Glu Gly Arg Val Thr 475 480 485	1671

gamma-1 C gene subcloned into pUC19 that had been linearized by digestion with Hind III and Sal I. Plasmid pICHu_γ-1M was digested with Hind III and Eco RI to isolate the 6 kb fragment encoding the human heavy chain constant 5 region. Plasmid pIC19H was linearized by digestion with Eco RI. The 1.65 kb PDGF β -R fragment, the 6 kb heavy chain constant region fragment and the linearized pIC19H were joined in a three part ligation. The resultant plasmid, pSDL111, was digested with Bam HI to isolate the 10 7.7 kb fragment. Plasmid pμPRE8 was linearized with Bgl II and was treated with calf intestinal phosphatase to prevent self-ligation. The 7.7 kb fragment and the linearized pμPRE8 were joined by ligation. A plasmid containing the insert in the proper orientation was 15 designated pSDL113 (Figure 8).

Plasmid pSDL113 is linearized by digestion with Cla I and is cotransfected with Pvu I-digested p416 into SP2/0-Ag14 by electroporation. Transfectants are selected in growth medium containing methotrexate.

20 Media from drug resistant clones are tested for the presence of secreted PDGF β -R analogs by immunoprecipitation using the method described in Example 12.B.

25 E. Cotransfection of pSDL113 with an Immunoglobulin Light Chain Gene

Plasmid pSDL113 is linearized by digestion with Cla I and was cotransfected with pICφ5V_kHuC_k-Neo, which encodes a neomycin resistance gene and a mouse/human 30 chimeric immunoglobulin light chain gene. The mouse immunoglobulin light chain gene was isolated from a lambda genomic DNA library constructed from the murine hybridoma cell line NR-ML-05 (Woodhouse et al., ibid.) using an oligonucleotide probe designed to span the V_k/J_k junction 35 (5' ACC GAA CGT GAG AGG AGT GCT ATA A 3'; Sequence ID Number 34). The human immunoglobulin light chain constant region gene was isolated as described in Example 12.B.

TTC GCC AAA GTG GAG GAG ACC ATC GCC GTG CGA TGC CTG GCT AAG AAT Phe Ala Lys Val Glu Glu Thr Ile Ala Val Arg Cys Leu Ala Lys Asn 490 495 500 505	1719
CTC CTT GGA GCT GAG AAC CGA GAG CTG AAG CTG GTG GCT CCC ACC CTG Leu Leu Gly Ala Glu Asn Arg Glu Leu Lys Leu Val Ala Pro Thr Leu 510 515 520	1767
CGT TCT GAA CTC ACG GTG GCT GCA GTC CTG GTG CTG TTG GTG ATT Arg Ser Glu Leu Thr Val Ala Ala Val Leu Val Leu Leu Val Ile 525 530 535	1815
G TG ATC ATC TCA CTT ATT GTC CTG GTT GTC ATT TGG AAA CAG AAA CCG Val Ile Ile Ser Leu Ile Val Leu Val Val Ile Trp Lys Gln Lys Pro 540 545 550	1863
AGG TAT GAA ATT CGC TGG AGG GTC ATT GAA TCA ATC AGC CCG GAT GGA Arg Tyr Glu Ile Arg Trp Arg Val Ile Glu Ser Ile Ser Pro Asp Gly 555 560 565	1911
CAT GAA TAT ATT TAT GTG GAC CCG ATG CAG CTG CCT TAT GAC TCA AGA His Glu Tyr Ile Tyr Val Asp Pro Met Gln Leu Pro Tyr Asp Ser Arg 570 575 580 585	1959
TGG GAG TTT CCA AGA GAT GGA CTA GTG CTT GGT CGG GTC TTG GGG TCT Trp Glu Phe Pro Arg Asp Gly Leu Val Leu Gly Arg Val Leu Gly Ser 590 595 600	2007
GGA GCG TTT GGG AAG GTG GTT GAA GGA ACA GCC TAT GGA TTA AGC CGG Gly Ala Phe Gly Lys Val Val Glu Gly Thr Ala Tyr Gly Leu Ser Arg 605 610 615	2055
TCC CAA CCT GTC ATG AAA GTT GCA GTG AAG ATG CTA AAA CCC ACG GCC Ser Gln Pro Val Met Lys Val Ala Val Lys Met Leu Lys Pro Thr Ala 620 625 630	2103
AGA TCC AGT GAA AAA CAA GCT CTC ATG TCT GAA CTG AAG ATA ATG ACT Arg Ser Ser Glu Lys Gln Ala Leu Met Ser Glu Leu Lys Ile Met Thr 635 640 645	2151
CAC CTG GGG CCA CAT TTG AAC ATT GTA AAC TTG CTG GGA GCC TGC ACC His Leu Gly Pro His Leu Asn Ile Val Asn Leu Leu Gly Ala Cys Thr 650 655 660 665	2199
AAG TCA GGC CCC ATT TAC ATC ATC ACA GAG TAT TGC TTC TAT GGA GAT Lys Ser Gly Pro Ile Tyr Ile Ile Thr Glu Tyr Cys Phe Tyr Gly Asp 670 675 680	2247
TTG GTC AAC TAT TTG CAT AAG AAT AGG GAT AGC TTC CTG AGC CAC CAC Leu Val Asn Tyr Leu His Lys Asn Arg Asp Ser Phe Leu Ser His His 685 690 695	2295
CCA GAG AAG CCA AAG AAA GAG CTG GAT ATC TTT GGA TTG AAC CCT GCT Pro Glu Lys Pro Lys Lys Glu Leu Asp Ile Phe Gly Leu Asn Pro Ala 700 705 710	2343

GAT GAA AGC ACA CGG AGC TAT GTT ATT TTA TCT TTT GAA AAC AAT GGT Asp Glu Ser Thr Arg Ser Tyr Val Ile Leu Ser Phe Glu Asn Asn Gly 715 720 725	2391
GAC TAC ATG GAC ATG AAG CAG GCT GAT ACT ACA CAG TAT GTC CCC ATG Asp Tyr Met Asp Met Lys Gln Ala Asp Thr Thr Gln Tyr Val Pro Met 730 735 740 745	2439
CTA GAA AGG AAA GAG GTT TCT AAA TAT TCC GAC ATC CAG AGA TCA CTC Leu Glu Arg Lys Glu Val Ser Lys Tyr Ser Asp Ile Gln Arg Ser Leu 750 755 760	2487
TAT GAT CGT CCA GCC TCA TAT AAG AAG AAA TCT ATG TTA GAC TCA GAA Tyr Asp Arg Pro Ala Ser Tyr Lys Lys Ser Met Leu Asp Ser Glu 765 770 775	2535
GTC AAA AAC CTC CTT TCA GAT GAT AAC TCA GAA GGC CTT ACT TTA TTG Val Lys Asn Leu Leu Ser Asp Asp Asn Ser Glu Gly Leu Thr Leu Leu 780 785 790	2583
GAT TTG TTG AGC TTC ACC TAT CAA GTT GCC CGA GGA ATG GAG TTT TTG Asp Leu Leu Ser Phe Thr Tyr Gln Val Ala Arg Gly Met Glu Phe Leu 795 800 805	2631
GCT TCA AAA AAT TGT GTC CAC CGT GAT CTG GCT GCT CGC AAC GTC CTC Ala Ser Lys Asn Cys Val His Arg Asp Leu Ala Ala Arg Asn Val Leu 810 815 820 825	2679
CTG GCA CAA GGA AAA ATT GTG AAG ATC TGT GAC TTT GGC CTG GCC AGA Leu Ala Gln Gly Lys Ile Val Lys Ile Cys Asp Phe Gly Leu Ala Arg 830 835 840	2727
GAC ATC ATG CAT GAT TCG AAC TAT GTG TCG AAA GGC AGT ACC TTT CTG Asp Ile Met His Asp Ser Asn Tyr Val Ser Lys Gly Ser Thr Phe Leu 845 850 855	2775
CCC GTG AAG TGG ATG GCT CCT GAG AGC ATC TTT GAC AAC CTC TAC ACC Pro Val Lys Trp Met Ala Pro Glu Ser Ile Phe Asp Asn Leu Tyr Thr 860 865 870	2823
ACA CTG AGT GAT GTC TGG TCT TAT GGC ATT CTG CTC TGG GAG ATC TTT Thr Leu Ser Asp Val Trp Ser Tyr Gly Ile Leu Leu Trp Glu Ile Phe 875 880 885	2871
TCC CTT GGT GGC ACC CCT TAC CCC GGC ATG ATG GTG GAT TCT ACT TTC Ser Leu Gly Gly Thr Pro Tyr Pro Gly Met Met Val Asp Ser Thr Phe 890 895 900 905	2919
TAC AAT AAG ATC AAG AGT GGG TAC CGG ATG GCC AAG CCT GAC CAC GCT Tyr Asn Lys Ile Lys Ser Gly Tyr Arg Met Ala Lys Pro Asp His Ala 910 915 920	2967

ACC AGT GAA GTC TAC GAG ATC ATG GTG AAA TGC TGG AAC AGT GAG CCG Thr Ser Glu Val Tyr Glu Ile Met Val Lys Cys Trp Asn Ser Glu Pro 925 930 935	3015
GAG AAG AGA CCC TCC TTT TAC CAC CTG AGT GAG ATT GTG GAG AAT CTG Glu Lys Arg Pro Ser Phe Tyr His Leu Ser Glu Ile Val Glu Asn Leu 940 945 950	3063
CTG CCT GGA CAA TAT AAA AAG AGT TAT GAA AAA ATT CAC CTG GAC TTC Leu Pro Gly Gln Tyr Lys Lys Ser Tyr Glu Lys Ile His Leu Asp Phe 955 960 965	3111
CTG AAG AGT GAC CAT CCT GCT GTG GCA CGC ATG CGT GTG GAC TCA GAC Leu Lys Ser Asp His Pro Ala Val Ala Arg Met Arg Val Asp Ser Asp 970 975 980 985	3159
AAT GCA TAC ATT GGT GTC ACC TAC AAA AAC GAG GAA GAC AAG CTG AAG Asn Ala Tyr Ile Gly Val Thr Tyr Lys Asn Glu Glu Asp Lys Leu Lys 990 995 1000	3207
GAC TGG GAG GGT GGT CTG GAT GAG CAG AGA CTG AGC GCT GAC AGT GGC Asp Trp Glu Gly Leu Asp Glu Gln Arg Leu Ser Ala Asp Ser Gly 1005 1010 1015	3255
TAC ATC ATT CCT CTG CCT GAC ATT GAC CCT GTC CCT GAG GAG GAG GAC Tyr Ile Ile Pro Leu Pro Asp Ile Asp Pro Val Pro Glu Glu Glu Asp 1020 1025 1030	3303
CTG GGC AAG AGG AAC AGA CAC AGC TCG CAG ACC TCT GAA GAG AGT GCC Leu Gly Lys Arg Asn Arg His Ser Ser Gln Thr Ser Glu Glu Ser Ala 1035 1040 1045	3351
ATT GAG ACG GGT TCC AGC AGT TCC ACC TTC ATC AAG AGA GAG GAC GAG Ile Glu Thr Gly Ser Ser Ser Ser Thr Phe Ile Lys Arg Glu Asp Glu 1050 1055 1060 1065	3399
ACC ATT GAA GAC ATC GAC ATG ATG GAC GAC ATC GGC ATA GAC TCT TCA Thr Ile Glu Asp Ile Asp Met Asp Asp Ile Gly Ile Asp Ser Ser 1070 1075 1080	3447
GAC CTG GTG GAA GAC AGC TTC CTG TAACTGGCGG ATTCAAGGGG TTCCCTCCAC Asp Leu Val Glu Asp Ser Phe Leu 1085	3501
TTCTGGGGCC ACCTCTGGAT CCCGTTAGA AAACCACTTT ATTGCAATGC GGAGGTTGAG	3561
AGGAGGACTT GGTTGATGTT TAAAGAGAAG TTCCCAGCCA AGGGCCTCGG GGAGCGTTCT	3621
AAATATGAAT GAATGGGATA TTTGAAATG AACTTGTCA GTGTTGCCCT TTGCAATGCC	3681
TCAGTAGCAT CTCAGTGGTG TGTGAAGTTT GGAGATAGAT GGATAAGGGA ATAATAGGCC	3741
ACAGAAGGTG AACTTGTGC TTCAAGGACA TTGGTGAGAG TCCAACAGAC ACAATTATA	3801
CTGCGACAGA ACTTCAGCAT TGTAATTATG TAAATAACTC TAACCAAGGC TGTGTTAGA	3861

TTGTATTAAC TATCTTCTTT GGACTTCTGA AGAGACCACT CAATCCATCC	3921
TCTTGAAACC TGATGTAGCT GCTGTTGAAC TTTTAAAGA AGTGCATGAA AAACCATTT	3981
TGAACCTTAA AAGGTACTGG TACTATAGCA TTTTGCTATC TTTTTAGTG TTAAAGAGAT	4041
AAAGAATAAT AAG	4054

(2) INFORMATION FOR SEQ ID NO:36:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1089 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

Met	Gly	Thr	Ser	His	Pro	Ala	Phe	Leu	Val	Leu	Gly	Cys	Leu	Leu	Thr
1															15
Gly	Leu	Ser	Leu	Ile	Leu	Cys	Gln	Leu	Ser	Leu	Pro	Ser	Ile	Leu	Pro
	20						25						30		
Asn	Glu	Asn	Glu	Lys	Val	Val	Gln	Leu	Asn	Ser	Ser	Phe	Ser	Leu	Arg
	35						40					45			
Cys	Phe	Gly	Glu	Ser	Glu	Val	Ser	Trp	Gln	Tyr	Pro	Met	Ser	Glu	Glu
	50					55						60			
Glu	Ser	Ser	Asp	Val	Glu	Ile	Arg	Asn	Glu	Glu	Asn	Asn	Ser	Gly	Leu
	65				70				75					80	
Phe	Val	Thr	Val	Leu	Glu	Val	Ser	Ser	Ala	Ser	Ala	Ala	His	Thr	Gly
	85					90							95		
Leu	Tyr	Thr	Cys	Tyr	Tyr	Asn	His	Thr	Gln	Thr	Glu	Glu	Asn	Glu	Leu
	100					105							110		
Glu	Gly	Arg	His	Ile	Tyr	Ile	Tyr	Val	Pro	Asp	Pro	Asp	Val	Ala	Phe
	115					120						125			
Val	Pro	Leu	Gly	Met	Thr	Asp	Tyr	Leu	Val	Ile	Val	Glu	Asp	Asp	Asp
	130					135						140			
Ser	Ala	Ile	Ile	Pro	Cys	Arg	Thr	Thr	Asp	Pro	Glu	Thr	Pro	Val	Thr
	145				150				155			160			
Leu	His	Asn	Ser	Glu	Gly	Val	Val	Pro	Ala	Ser	Tyr	Asp	Ser	Arg	Gln
	165					170						175			

Gly Phe Asn Gly Thr Phe Thr Val Gly Pro Tyr Ile Cys Glu Ala Thr
 180 185 190
 Val Lys Gly Lys Lys Phe Gln Thr Ile Pro Phe Asn Val Tyr Ala Leu
 195 200 205
 Lys Ala Thr Ser Glu Leu Asp Leu Glu Met Glu Ala Leu Lys Thr Val
 210 215 220
 Tyr Lys Ser Gly Glu Thr Ile Val Val Thr Cys Ala Val Phe Asn Asn
 225 230 235 240
 Glu Val Val Asp Leu Gln Trp Thr Tyr Pro Gly Glu Val Lys Gly Lys
 245 250 255
 Gly Ile Thr Ile Leu Glu Glu Ile Lys Val Pro Ser Ile Lys Leu Val
 260 265 270
 Tyr Thr Leu Thr Val Pro Glu Ala Thr Val Lys Asp Ser Gly Asp Tyr
 275 280 285
 Glu Cys Ala Ala Arg Gln Ala Thr Arg Glu Val Lys Glu Met Lys Lys
 290 295 300
 Val Thr Ile Ser Val His Glu Lys Gly Phe Ile Glu Ile Lys Pro Thr
 305 310 315 320
 Phe Ser Gln Leu Glu Ala Val Asn Leu His Glu Val Lys His Phe Val
 325 330 335
 Val Glu Val Arg Ala Tyr Pro Pro Pro Arg Ile Ser Trp Leu Lys Asn
 340 345 350
 Asn Leu Thr Leu Ile Glu Asn Leu Thr Glu Ile Thr Thr Asp Val Glu
 355 360 365
 Lys Ile Gln Glu Ile Arg Tyr Arg Ser Lys Leu Lys Leu Ile Arg Ala
 370 375 380
 Lys Glu Glu Asp Ser Gly His Tyr Thr Ile Val Ala Gln Asn Glu Asp
 385 390 395 400
 Ala Val Lys Ser Tyr Thr Phe Glu Leu Leu Thr Gln Val Pro Ser Ser
 405 410 415
 Ile Leu Asp Leu Val Asp Asp His His Gly Ser Thr Gly Gly Gln Thr
 420 425 430
 Val Arg Cys Thr Ala Glu Gly Thr Pro Leu Pro Asp Ile Glu Trp Met
 435 440 445
 Ile Cys Lys Asp Ile Lys Lys Cys Asn Asn Glu Thr Ser Trp Thr Ile
 450 455 460

Leu Ala Asn Asn Val Ser Asn Ile Ile Thr Glu Ile His Ser Arg Asp
 465 470 475 480
 Arg Ser Thr Val Glu Gly Arg Val Thr Phe Ala Lys Val Glu Glu Thr
 485 490 495
 Ile Ala Val Arg Cys Leu Ala Lys Asn Leu Leu Gly Ala Glu Asn Arg
 500 505 510
 Glu Leu Lys Leu Val Ala Pro Thr Leu Arg Ser Glu Leu Thr Val Ala
 515 520 525
 Ala Ala Val Leu Val Leu Val Ile Val Ile Ile Ser Leu Ile Val
 530 535 540
Leu Val Val Ile Trp Lys Gln Lys Pro Arg Tyr Glu Ile Arg Trp Arg
545 550 555 560
 Val Ile Glu Ser Ile Ser Pro Asp Gly His Glu Tyr Ile Tyr Val Asp
 565 570 575
 Pro Met Gln Leu Pro Tyr Asp Ser Arg Trp Glu Phe Pro Arg Asp Gly
 580 585 590
 Leu Val Leu Gly Arg Val Leu Gly Ser Gly Ala Phe Gly Lys Val Val
 595 600 605
 Glu Gly Thr Ala Tyr Gly Leu Ser Arg Ser Gln Pro Val Met Lys Val
 610 615 620
 Ala Val Lys Met Leu Lys Pro Thr Ala Arg Ser Ser Glu Lys Gln Ala
 625 630 635 640
 Leu Met Ser Glu Leu Lys Ile Met Thr His Leu Gly Pro His Leu Asn
 645 650 655
 Ile Val Asn Leu Leu Gly Ala Cys Thr Lys Ser Gly Pro Ile Tyr Ile
 660 665 670
 Ile Thr Glu Tyr Cys Phe Tyr Gly Asp Leu Val Asn Tyr Leu His Lys
 675 680 685
 Asn Arg Asp Ser Phe Leu Ser His His Pro Glu Lys Pro Lys Lys Glu
 690 695 700
 Leu Asp Ile Phe Gly Leu Asn Pro Ala Asp Glu Ser Thr Arg Ser Tyr
 705 710 715 720
 Val Ile Leu Ser Phe Glu Asn Asn Gly Asp Tyr Met Asp Met Lys Gln
 725 730 735
 Ala Asp Thr Thr Gln Tyr Val Pro Met Leu Glu Arg Lys Glu Val Ser
 740 745 750

Lys Tyr Ser Asp Ile Gln Arg Ser Leu Tyr Asp Arg Pro Ala Ser Tyr
 755 760 765
 Lys Lys Lys Ser Met Leu Asp Ser Glu Val Lys Asn Leu Leu Ser Asp
 770 775 780
 Asp Asn Ser Glu Gly Leu Thr Leu Leu Asp Leu Leu Ser Phe Thr Tyr
 785 790 795 800
 Gln Val Ala Arg Gly Met Glu Phe Leu Ala Ser Lys Asn Cys Val His
 805 810 815
 Arg Asp Leu Ala Ala Arg Asn Val Leu Leu Ala Gln Gly Lys Ile Val
 820 825 830
 Lys Ile Cys Asp Phe Gly Leu Ala Arg Asp Ile Met His Asp Ser Asn
 — 835 840 845
 Tyr Val Ser Lys Gly Ser Thr Phe Leu Pro Val Lys Trp Met Ala Pro
 850 855 860
 Glu Ser Ile Phe Asp Asn Leu Tyr Thr Thr Leu Ser Asp Val Trp Ser
 865 870 875 880
 Tyr Gly Ile Leu Leu Trp Glu Ile Phe Ser Leu Gly Gly Thr Pro Tyr
 885 890 895
 Pro Gly Met Met Val Asp Ser Thr Phe Tyr Asn Lys Ile Lys Ser Gly
 900 905 910
 Tyr Arg Met Ala Lys Pro Asp His Ala Thr Ser Glu Val Tyr Glu Ile
 915 920 925
 Met Val Lys Cys Trp Asn Ser Glu Pro Glu Lys Arg Pro Ser Phe Tyr
 930 935 940
 His Leu Ser Glu Ile Val Glu Asn Leu Leu Pro Gly Gln Tyr Lys Lys
 945 950 955 960
 Ser Tyr Glu Lys Ile His Leu Asp Phe Leu Lys Ser Asp His Pro Ala
 965 970 975
 Val Ala Arg Met Arg Val Asp Ser Asp Asn Ala Tyr Ile Gly Val Thr
 980 985 990
 Tyr Lys Asn Glu Glu Asp Lys Leu Lys Asp Trp Glu Gly Leu Asp
 995 1000 1005
 Glu Gln Arg Leu Ser Ala Asp Ser Gly Tyr Ile Ile Pro Leu Pro Asp
 1010 1015 1020
 Ile Asp Pro Val Pro Glu Glu Asp Leu Gly Lys Arg Asn Arg His
 1025 1030 1035 1040

Ser Ser Gln Thr Ser Glu Glu Ser Ala Ile Glu Thr Gly Ser Ser Ser
1045 1050 1055

Ser Thr Phe Ile Lys Arg Glu Asp Glu Thr Ile Glu Asp Ile Asp Met
1060 1065 1070

Met Asp Asp Ile Gly Ile Asp Ser Ser Asp Leu Val Glu Asp Ser Phe
1075 1080 1085

Leu

Claims

1. A method for producing a secreted, biologically active dimerized polypeptide fusion, comprising:

introducing into a eukaryotic host cell a DNA construct comprising a transcriptional promoter operatively linked to a secretory signal sequence followed downstream by and in proper reading frame with a DNA sequence encoding a non-immunoglobulin polypeptide requiring dimerization for biological activity joined to a dimerizing protein;

growing said host cell in an appropriate growth medium under physiological conditions to allow the secretion of a dimerized polypeptide fusion encoded by said DNA sequence; and

isolating said dimerized polypeptide fusion from said host cell.

2. A method for producing a secreted, biologically active dimerized polypeptide fusion, comprising:

introducing into a eukaryotic host cell a first DNA construct comprising a transcriptional promoter operatively linked to a first secretory signal sequence followed downstream by and in proper reading frame with a first DNA sequence encoding a non-immunoglobulin polypeptide requiring dimerization for biological activity joined to an immunoglobulin light chain constant region;

introducing into said host cell a second DNA construct comprising a transcriptional promoter operatively linked to a second secretory signal sequence followed downstream by and in proper reading frame with a second DNA sequence encoding an immunoglobulin heavy chain constant region domain selected from the group consisting of C_H1, C_H2, C_H3, and C_H4;

growing said host cell in an appropriate growth medium under physiological conditions to allow the secretion of a biologically active dimerized polypeptide fusion encoded by said first and second DNA sequences; and

isolating said biologically active dimerized polypeptide fusion from said host cell.

3. The method of claim 2 wherein said second DNA sequence further encodes an immunoglobulin hinge region and wherein said hinge region is joined to said immunoglobulin heavy chain constant region.

4. The method of claim 2 wherein said second DNA sequence further encodes an immunoglobulin variable region and wherein said variable region is joined upstream of and in proper reading frame with said immunoglobulin heavy chain constant region domain.

5. The method of claim 2 wherein said host cell is a fungal cell or a cultured mammalian cell.

6. The method of claim 2 wherein said host cell is a cultured rodent myeloma cell line.

7. The method of claim 2 wherein said non-immunoglobulin polypeptide requiring dimerization for biological activity is selected from the group consisting of a polypeptide comprising the amino acid sequence of Figure 1 (Sequence ID Numbers 1 and 2) from isoleucine, number 29, to lysine, number 531, a polypeptide comprising the amino acid sequence of Figure 1 (Sequence ID Numbers 1 and 2) from isoleucine, number 29, to methionine, number 441, and a polypeptide comprising the amino acid sequence of Figure 11 (Sequence ID Numbers 35 and 36) from glutamine, number 24 to glutamic acid, number 524.

8. A method for producing a secreted, biologically active dimerized polypeptide fusion, comprising:

introducing into a eukaryotic host cell a first DNA construct comprising a transcriptional promoter operatively linked to a first secretory signal sequence followed

downstream by and in proper reading frame with a first DNA sequence encoding a non-immunoglobulin polypeptide requiring dimerization for biological activity joined to an immunoglobulin heavy chain constant region domain selected from the group consisting of C_H1, C_H2, C_H3, and C_H4;

introducing into said host cell a second DNA construct comprising a transcriptional promoter operatively linked to a second secretory signal sequence followed downstream by and in proper reading frame with a second DNA sequence encoding an immunoglobulin light chain constant region;

growing said host cell in an appropriate growth medium under physiological conditions to allow the secretion of a biologically active dimerized polypeptide fusion encoded by said first and second DNA sequences; and

isolating said biologically active dimerized polypeptide fusion from said host cell.

9. The method of claim 8 wherein said first DNA sequence further encodes an immunoglobulin hinge region and wherein said hinge region is joined to said immunoglobulin constant region.

10. The method of claim 8 wherein said second DNA sequence further encodes an immunoglobulin variable region and wherein said variable region is joined upstream of and in proper reading frame with said immunoglobulin light chain constant region domain.

11. The method of claim 8 wherein said host cell is a fungal cell or a cultured mammalian cell.

12. The method of claim 8 wherein said host cell is a cultured rodent myeloma cell line.

13. The method of claim 8 wherein said non-immunoglobulin polypeptide requiring dimerization for

biological activity is selected from the group consisting of a polypeptide comprising the amino acid sequence of Figure 1 (Sequence ID Numbers 1 and 2) from isoleucine, number 29, to lysine, number 531 a polypeptide comprising the amino acid sequence of Figure 1 (Sequence ID Numbers 1 and 2) from isoleucine, number 29, to methionine, number 441, and a polypeptide comprising the amino acid sequence of Figure 11 (Sequence ID Numbers 35 and 36) from glutamine, number 24 to glutamic acid, number 524.

14. A method for producing a secreted receptor analog, comprising:

introducing into a eukaryotic host cell a DNA construct comprising a transcriptional promoter operatively linked to at least one secretory signal sequence followed downstream by and in proper reading frame with a DNA sequence encoding a ligand-binding domain of a receptor requiring dimerization for biological activity joined to a dimerizing protein;

growing said host cell in an appropriate growth medium under physiological conditions to allow the secretion of a receptor analog encoded by said DNA sequence; and

isolating said receptor analog from said host cell.

15. A method for determining the presence of PDGF or isoforms thereof in a biological sample, comprising:

incubating a polypeptide comprising a PDGF receptor analog fused to a dimerizing protein with a biological sample suspected of comprising PDGF or an isoform thereof under physiological conditions to allow the formation of receptor/ligand complexes; and

detecting the presence of the receptor/ligand complexes as an indication of the presence of human PDGF or an isoform thereof.

16. The method of claim 15 wherein the polypeptide is tagged with a label selected from the group consisting of radionuclides, fluorophores, enzymes, and luminescers.

17. The method of claim 15 wherein the biological sample is selected from the group consisting of blood, urine, plasma, serum, platelet and other cell lysates, platelet releasates, cell suspensions, cell-conditioned culture media and chemically or physically separated portions thereof.

18. The method of claim 15 wherein said human PDGF receptor analog is selected from the group consisting of the amino acid sequence of Figure 1 (Sequence ID Numbers 1 and 2) from isoleucine, number 29, to methionine, number 441, joined to a dimerizing protein, the amino acid sequence of Figure 1 (Sequence ID Numbers 1 and 2) from isoleucine, number 29, to lysine, number 531, joined to a dimerizing protein and the amino acid sequence of Figure 11 (Sequence ID Numbers 35 and 36) from glutamine, number 24 to glutamic acid, number 524, joined to a dimerizing protein.

19. The method of claim 15 wherein said dimerizing protein comprises at least a portion of a protein selected from the group consisting of an immunoglobulin light chain, an immunoglobulin heavy chain and yeast invertase, wherein said portion associates as a dimer in a covalent or a noncovalent manner.

20. A method for producing a secreted PDGF receptor analog, comprising:

introducing into a eukaryotic host cell a DNA construct comprising a transcriptional promoter operatively linked to a secretory signal sequence followed downstream in proper reading frame by a DNA sequence encoding a ligand-binding domain of a PDGF receptor;

growing said host cell in an appropriate growth medium under physiological conditions to allow the secretion of a PDGF receptor analog encoded by said DNA sequence; and isolating said PDGF receptor analog from said host cell.

21. A method for producing a secreted PDGF receptor analog, comprising:

introducing into a cultured rodent myeloma cell a DNA construct comprising a transcriptional promoter operatively linked to a PDGF receptor signal sequence followed downstream by and in proper reading frame with a DNA sequence encoding the amino acid sequence of Figure 11 (Sequence ID Numbers 35 and 36) from glutamine, number 24, to glutamic acid, number 524, joined to a dimerizing protein, wherein said dimerizing protein is an immunoglobulin constant region selected from the group consisting of C_H1, C_H2, C_H3, C_H4 and C_K joined to an immunoglobulint hinge region;

growing said cultured rodent myeloma cell in an appropriate growth medium under physiological conditions to allow the secretion of a PDGF receptor analog encoded by said DNA sequence; and

isolating the PDGF receptor analog from said cultured rodent myeloma cell.

22. A method for producing a secreted PDGF receptor analog, comprising:

introducing into a cultured rodent myeloma cell a first DNA construct comprising a transcriptional promoter operatively linked to a PDGF receptor signal sequence followed downstream by and in proper reading frame with a first DNA sequence encoding the amino acid sequence of Figure 1 (Sequence ID Numbers 1 and 2) from isoleucine, number 29, to lysine, number 531 joined to an immunoglobulin light chain constant region;

introducing into said cultured rodent myeloma cell a second DNA construct comprising a transcriptional promoter

operatively linked to a PDGF receptor signal sequence followed downstream by and in proper reading frame with a second DNA sequence encoding the amino acid sequence of Figure 1 (Sequence ID Numbers 1 and 2) from isoleucine number 29, to lysine, number 531 joined to an immunoglobulin heavy chain constant region domain selected from the group consisting of C_H1, C_H2, C_H3, and C_H4 joined to an immunoglobulin hinge region;

growing said cultured rodent myeloma cell in an appropriate growth medium under physiological conditions to allow the secretion of a PDGF receptor analog encoded by said first and second DNA sequences; and

isolating said PDGF receptor analog from said cultured rodent myeloma cell.

23. A method for producing a secreted PDGF receptor analog, comprising:

introducing into a cultured rodent myeloma cell a first DNA construct comprising a mouse V_H promoter operatively linked to a PDGF receptor signal sequence followed downstream of and in proper reading frame with a DNA sequence encoding the amino acid sequence of Figure 11 (Sequence ID Numbers 35 and 36) from glutamine, number 24 to glutamic acid, number 524, joined to an immunoglobulin heavy chain constant region domain selected from the group consisting of C_H1, C_H2, C_H3 and C_H4 joined to an immunoglobulin hinge region;

introducing into said cultured rodent myeloma cell a second DNA construct comprising a mouse V_L promoter operatively linked to a PDGF receptor signal sequence followed downstream of and in proper reading frame with a DNA sequence encoding the amino acid sequence of Figure 11 (Sequence ID Numbers 35 and 36) from glutamine, number 24 to glutamic acid, number 524, joined to an immunoglobulin light chain constant region;

growing said cultured rodent myeloma cell in an appropriate growth medium under physiological conditions to

allow the secretion of a PDGF receptor analog encoded by said first and second DNA sequences; and

isolating said PDGF receptor analog from said cultured myeloma cell.

24. A method for producing a secreted PDGF receptor analog, comprising:

introducing into a cultured rodent myeloma cell a first DNA construct comprising a mouse V_H promoter operatively linked to a PDGF receptor signal sequence followed downstream of and in proper reading frame with a DNA sequence encoding the amino acid sequence of Figure 1 (Sequence ID Numbers 1 and 2) from isoleucine number 29, to lysine, number 531, joined to an immunoglobulin heavy chain constant region domain selected from the group consisting of C_{H1} , C_{H2} , C_{H3} and C_{H4} joined to an immunoglobulin hinge region;

introducing into said cultured rodent myeloma cell a second DNA construct comprising a mouse V_k promoter operatively linked to a PDGF receptor signal sequence followed downstream of and in proper reading frame with a DNA sequence encoding the amino acid sequence of Figure 11 (Sequence ID Numbers 35 and 36) from glutamine, number 24 to glutamic acid, number 524, joined to an immunoglobulin light chain constant region;

growing said cultured rodent myeloma cell in an appropriate growth medium under physiological conditions to allow the secretion of a PDGF receptor analog encoded by said first and second DNA sequences; and

isolating said PDGF receptor analog from said cultured myeloma cell.

25. A method for producing a secreted PDGF receptor analog, comprising:

introducing into a cultured rodent myeloma cell a first DNA construct comprising a mouse V_H promoter operatively linked to a PDGF receptor signal sequence followed downstream of and in proper reading frame with a DNA sequence encoding

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the amino acid sequence of Figure 11 (Sequence ID Numbers 35 and 36) from glutamine, number 24 to glutamic acid, number 524, joined to an immunoglobulin heavy chain constant region domain selected from the group consisting of C_H1, C_H2, C_H3 and C_H4 joined to an immunoglobulin hinge region;

introducing into said cultured rodent myeloma cell a second DNA construct comprising a mouse V_k promoter operatively linked to a PDGF receptor signal sequence followed downstream of and in proper reading frame with a DNA sequence encoding the amino acid sequence of Figure 1 (Sequence ID Numbers 1 and 2) from isoleucine number 29, to lysine, number 531, joined to an immunoglobulin light chain constant region;

growing said cultured rodent myeloma cell in an appropriate growth medium under physiological conditions to allow the secretion of a PDGF receptor analog encoded by said first and second DNA sequences; and

isolating said PDGF receptor analog from said cultured myeloma cell.

26. A method for determining the presence of PDGF or an isoform thereof in a biological sample comprising the steps of:

incubating a polypeptide comprising a PDGF receptor analog joined to a dimerizing protein with a biological sample suspected of containing PDGF or an isoform thereof under conditions that allow the formation of receptor/ligand complexes; and

detecting the presence of receptor/ligand complexes, and therefrom determining the presence of human PDGF or an isoform thereof.

27. The method according to claim 26 wherein said biological sample is selected from the group consisting of blood, urine, plasma, serum, platelet and other cell lysates, platelet releasates, cell suspensions, cell-conditioned culture media, and chemically or physically separated portions thereof.

28. A method for purifying PDGF or an isoform thereof from a sample, comprising:

immobilizing a polypeptide comprising a PDGF receptor analog fused to a dimerizing protein on a substrate;

contacting a sample comprising PDGF or an isoform thereof with the immobilized polypeptide under conditions such that the PDGF or isoform thereof binds to the polypeptide; and eluting the PDGF or isoform thereof from the polypeptide.

METHODS OF PRODUCING SECRETED RECEPTOR ANALOGS AND BIOLOGICALLY ACTIVE DIMERIZED POLYPEPTIDE FUSIONS

Abstract of the Disclosure

Methods for producing secreted receptor analogs and biologically active peptide dimers are disclosed. The methods for producing secreted receptor analogs and biologically active peptide dimers utilize a DNA sequence encoding a receptor analog or a peptide requiring dimerization for biological activity joined to a dimerizing protein. The receptor analog includes a ligand-binding domain. Polypeptides comprising essentially the extracellular domain of a human PDGF receptor fused to dimerizing proteins, the portion being capable of binding human PDGF or an isoform thereof, are also disclosed. The polypeptides may be used within methods for determining the presence of and for purifying human PDGF or isoforms thereof.

10	20	30	40	50	60	70
CCCTCAGCCC	TGCTGCCAG	CACGAGCCTG	TGCTGCCCT	GCCCAACGCA	GACAGCCAGA	CCCAGGGCGG
80	90	100	110	120	130	140
CCCCCTCTGGC	GGCTCTGCTC	CTCCCGAAGG	ATGCTTGGGG	AGTGAGGCGA	AGCTGGGCCG	TCCTCTCCCC
150	160	170	180	190	200	210
TACAGCAGCC	CCCTCCTCC	ATCCCTCTGT	TCTCCTGAGC	CTTCAGGAGC	CTGCACCAGT	CCTGCCTGTC
220	230	240	250	260	270	280
CTTCTACTCA	GCTGTTACCC	ACTCTGGGAC	CAGCAGTCTT	TCTGATAACT	GGGAGAGGGC	AGTAAGGAGG
290	300	310	320	330	340	350
ACTTCCCTGGA	GGGGGTGACT	GTCCAGAGCC	TGGAACTGTG	CCCACACCAAG	AAGCCATCAG	CAGCAAGGAC
359	368	377	386	395	404	
ACC ATG CGG CTT CCG GGT GCG ATG CCA GCT CTG GCC CTC AAA GGC GAG CTG CTG	M R L P G A M P A L A L K G E L L					17
413	422	431	440	449	458	
TTG CTG TCT-CTC CTG CTT CTG GAA CCA CAG ATC TCT CAG GGC CTG GTC GTC	L L S L L L E P Q I S Q G L V V					35
467	476	485	494	503	512	
ACA CCC CCG GGG CCA GAG CTT GTC CTC AAT GTC TCC AGC ACC TTC GTT CTG ACC	T P P G P E L V L N V S S T F V L T					53
521	530	539	548	557	566	
TGC TCG GGT TCA GCT CCG GTG GTG TGG GAA CGG ATG TCC CAG GAG CCC CCA CAG	C S G S A P V V W E R M S Q E P P Q					71
575	584	593	602	611	620	
GAA ATG GCC AAG GCC CAG GAT GGC ACC TTC TCC AGC GTG CTC ACA CTG ACC AAC	E M A K A Q D G T F S S V L T L T N					89
629	638	647	656	665	674	
CTC ACT GGG CTA GAC ACG GGA GAA TAC TTT TGC ACC CAC AAT GAC TCC CGT GGA	L T G L D T G E Y F C T H N D S R G					107
683	692	701	710	719	728	
CTG GAG ACC GAT GAG CGG AAA CGG CTC TAC ATC TTT GTG CCA GAT CCC ACC GTG	L E T D E R K R L Y I F V P D P T V					125
737	746	755	764	773	782	
GGC TTC CTC CCT AAT GAT GCC GAG GAA CTA TTC ATC TTT CTC ACG GAA ATA ACT	S F L P N D A E E L F I F L T E I T					143
791	800	809	818	827	836	
SAG ATC ACC ATT CCA TGC CGA GTA ACA GAC CCA CAG CTG GTG GTG ACA CTG CAC	E I T I P C R V T D P Q L V V T L H					161
845	854	863	872	881	890	
SAG AAG AAA GGG GAC GTT GCA CTG CCT GTC CCC TAT GAT CAC CAA CGT GGC TTT	S K K G D V A L P V P Y D H Q R G F					179
899	908	917	926	935	944	
TCT GGT ATC TTT GAG GAC AGA AGC TAC ATC TGC AAA ACC ACC ATT GGG GAC AGG	S G I F E D R S Y I C K T T I G D R					197

953	962	971	980	989	998	
GAG GTG GAT TCT GAT GCC TAC TAT GTC TAC AGA CTC CAG GTG TCA TCC ATC AAC						
E V D S D A Y Y V Y R L Q V S S I N						215
1007	1016	1025	1034	1043	1052	
GTC TCT GTG AAC GCA GTG CAG ACT GTG GTC CGC CAG GGT GAG AAC ATC ACC CTC						
V S V N A V Q T V V R Q G E N I T L						233
1061	1070	1079	1088	1097	1106	
ATG TGC ATT GTG ATC GGG AAT GAG GTG GTC AAC TTC GAG TGG ACA TAC CCC CGC						
M C I V I G N E V V N F E W T Y P R						251
1115	1124	1133	1142	1151	1160	
AAA GAA AGT GGG CGG CTG GTG GAG CCG GTG ACT GAC TTC CTC TTG GAT ATG CCT						
X E S G R L V E P V T D F L L D M P						269
1169	1178	1187	1196	1205	1214	
TAC CAC ATC CGC TCC ATC CTG CAC ATC CCC AGT GCC GAG TTA GAA GAC TCG GGG						
Y H I R S I L H I P S A E L E D S G						287
1223	1232	1241	1250	1259	1268	
ACC TAC ACC TGC AAT GTG ACG GAG AGT GTG AAT GAC CAT CAG GAT GAA AAG GCC						
T Y T C N V T E S V N D H Q D E K A						305
1277	1286	1295	1304	1313	1322	
ATC AAC ATC ACC GTG GTT GAG AGC GGC TAC GTG CGG CTC CTG GGA GAG GTG GGC						
I N I T V V E S G Y V R L L G E V G						323
1331	1340	1349	1358	1367	1376	
ACA CTA CAA TTT GCT GAG CTG CAT CGG AGC CGG ACA CTG CAG GTA GTG TTC GAG						
T L Q F A E L H R S R T L Q V V F E						341
1385	1394	1403	1412	1421	1430	
GCC TAC CCA CCG CCC ACT GTC CTG TGG TTC AAA GAC AAC CGC ACC CTG GGC GAC						
A Y P P T V L W F K D N R T L G D						359
1439	1448	1457	1466	1475	1484	
TCC AGC GCT GGC GAA ATC GCC CTG TCC ACG CGC AAC GTG TCG GAG ACC CGG TAT						
S S A G E I A L S T R N V S E T R Y						377
1493	1502	1511	1520	1529	1538	
GTG TCA GAG CTG ACA CTG GTT CGC GTG AAG GTG GCA GAG GCT GGC CAC TAC ACC						
V S E L T L V R V K V A E A G H Y T						391
1547	1556	1565	1574	1583	1592	
ATG CGG GCC TTC CAT GAG GAT GCT GAG GTC CAG CTC TCC TTC CAG CTA CAG ATC						
M R A F H E D A E V Q L S F Q L Q I						413
1601	1610	1619	1628	1637	1646	
AAT GTC CCT GTC CGA GTG CTG GAG CTA AGT GAG AGC CAC CCT GAC AGT GGG GAA						
N V P V R V L E L S E S H P D S G E						431
1655	1664	1673	1682	1691	1700	
CAG ACA GTC CGC TGT CGT GGC CGG GGC ATG CCC CAG CCG AAC ATC ATC TGG TCT						
Q T V R C R G R G M P Q P N I I W S						449
1709	1718	1727	1736	1745	1754	
GCC TGC AGA GAC CTC AAA AGG TGT CCA CGT GAG CTG CCG CCC ACG CTG CTG GGG						
A C R D L K R C P R E L P P T L L G						467

1763	1772	1781	1790	1799	1808	
AAC AGT TCC GAA GAG GAG AGC CAG CTG GAG ACT AAC GTG ACG TAC TGG GAG GAG						
N S S E E E S Q L E T N V T Y W E E						485
1817	1826	1835	1844	1853	1862	
GAG CAG GAG TTT GAG GTG GTG AGC ACA CTG CGT CTG CAG CAC GTG GAT CGG CCA						
E Q E F E V V S T L R L Q H V D R P						503
1871	1880	1889	1898	1907	1916	
CTG TCG GTG CGC TGC ACG CTG CGC AAC GCT GTG GGC CAG GAC ACG CAG GAG GTC						
L S V R C T L R N A V G Q D T Q E V						521
1925	1934	1943	1952	1961	1970	
ATC GTG GTG CCA CAC TCC TTG CCC TTT AAG GTG GTG GTG ATC TCA GCC ATC CTG						
I V V P H S L P F K V V V I S A I L						539
1979	1988	1997	2006	2015	2024	
GCC CTG GTG GTG CTC ACC ATC ATC TCC CTT ATC ATC CTC ATC ATG CTT TGG CAG						
A L V V L T I I S L I I L I M L W Q						557
2033	2042	2051	2060	2069	2078	
AAG AAG CCA CGT TAC GAG ATC CGA TGG AAG GTG ATT GAG TCT GTG AGC TCT GAC						
K K P R Y E I R W X V I E S V S S D						575
2087	2096	2105	2114	2123	2132	
GGC CAT GAG TAC ATC TAC GTG GAC CCC ATG CAG CTG CCC TAT GAC TCC ACG TGG						
S H E Y I Y V D P M Q L P Y D S T W						593
2141	2150	2159	2168	2177	2186	
GGG CTG CCG CGG GAC CAG CTT GTG CTG GGA CGC ACC CTC GGC TCT GGG GCC TTT						
E L P R D Q L V L G R T L G S G A F						611
2195	2204	2213	2222	2231	2240	
GGG CAG GTG GTG GAG GCC ACG GCT CAT GGC CTG AGC CAT TCT CAG GCC ACG ATG						
S Q V V E A T A H G L S H S Q A T M						629
2249	2258	2267	2276	2285	2294	
AAA GTG GCC GTC AAG ATG CTT AAA TCC ACA GCA CGC AGC AGT GAG AAG CAA GCC						
K V A V K M L K S T A R S S E K Q A						647
2303	2312	2321	2330	2339	2348	
CTT ATG TCG GAG CTG AAG ATC ATG AGT CAC CTT GGG CCC CAC CTG AAC GTG GTC						
J M S E L K I M S H L G P H L N V V						665
2357	2366	2375	2384	2393	2402	
AAC CTG TTG GGG GCC TGC ACC AAA GGA GGA CCC ATC TAT ATC ATC ACT GAG TAC						
I L L G A C T K G G P I Y I I T E Y						683
2411	2420	2429	2438	2447	2456	
GGC CGC TAC GGA GAC CTG GTG GAC TAC CTG CAC CGC AAC AAA CAC ACC TTC CTG						
: R Y G D L V D Y L H R N K H T F L						701
2465	2474	2483	2492	2501	2510	
AG CAC CAC TCC GAC AAG CGC CGC CCG CCC AGC GCG GAG CTC TAC ACC AAT GCT						
I H H S D K R R P P S A E L Y S N A						719
2519	2528	2537	2546	2555	2564	
TG CCC GTT GGG CTC CCC CTG CCC AGC CAT GTG TCC TTG ACC GGG GAG AGC GAC						
. P V G L P L P S H V S L T G E S D						737

2573	2582	2591	2600	2609	2618	
GGT GGC TAC ATG GAC ATG AGC AAG GAC GAG TCG GTG GAC TAT GTG CCC ATG CTG	G G Y M D M S K D E S V D Y V P M L	755				
2627	2636	2645	2654	2663	2672	
GAC ATG AAA GGA GAC GTC AAA TAT GCA GAC ATC GAG TCC TCC AAC TAC ATG GCC	D M K G D V K Y A D I E S S N Y M A	773				
2681	2690	2699	2708	2717	2726	
CCT TAC GAT AAC TAC GTT CCC TCT GCC CCT GAG AGG ACC TGC CGA GCA ACT TTG	P Y D N Y V P S A P E R T C R A T L	791				
2735	2744	2753	2762	2771	2780	
ATC AAC GAG TCT CCA GTG CTA AGC TAC ATG GAC CTC GTG GGC TTC AGC TAC CAG	I N E S P V L S Y M D L V G F S Y Q	809				
2789	2798	2807	2816	2825	2834	
GTG GCC AAT GGC ATG GAG TTT CTG GCC TCC AAG AAC TGC GTC CAC AGA GAC CTG	V A N G M E F L A S K N C V H R D L	827				
2843	2852	2861	2870	2879	2888	
GCG GCT AGG AAC GTG CTC ATC TGT GAA GGC AAG CTG GTC AAG ATC TGT GAC TTT	A A R N V L I C E G K L V K I C D F	845				
2897	2906	2915	2924	2933	2942	
GGC CTG GCT CGA GAC ATC ATG CGG GAC TCG AAT TAC ATC TCC AAA GGC AGC ACC	G L A R D I M R D S N Y I S K G S T	863				
2951	2960	2969	2978	2987	2996	
TTT TTG CCT TTA AAG TGG ATG GCT CCG GAG AGC ATC TTC AAC AGC CTC TAC ACC	F L P L K W M A P E S I F N S L Y T	881				
3005	3014	3023	3032	3041	3050	
ACC CTG AGC GAC GTG TGG TCC TTC GGG ATC CTG CTC TGG GAG ATC TTC ACC TTG	F L S D V W S F G I L L W E I F T L	899				
3059	3068	3077	3086	3095	3104	
GGT GGC ACC CCT TAC CCA GAG CTG CCC ATG AAC GAG CAG TTC TAC AAT GCC ATC	S G T P Y P E L P M N E Q F Y N A I	917				
3113	3122	3131	3140	3149	3158	
AAA CGG GGT TAC CGC ATG GCC CAG CCT GCC CAT GCC TCC GAC GAG ATC TAT GAG	K R G Y R M A Q P A H A S D E I Y E	935				
3167	3176	3185	3194	3203	3212	
ATC ATG CAG AAG TGC TGG GAA GAG AAG TTT GAG ATT CGG CCC CCC TTC TCC CAG	I M Q K C W E E K F E I R P P F S Q	953				
3221	3230	3239	3248	3257	3266	
CTG GTG CTG CTT CTC GAG AGA CTG TTG GGC GAA GGT TAC AAA AAG AAG TAC CAG	J V L L E R L L G E G Y K K K Y Q	971				
3275	3284	3293	3302	3311	3320	
AGG GTG GAT GAG GAG TTT CTG AGG AGT GAC CAC CCA GCC ATC CTT CGG TCC CAG	{ V D E E F L R S D H P A I L R S Q	989				
3329	3338	3347	3356	3365	3374	
CCC CGC TTG CCT GGG TTC CAT GGC CTC CGA TCT CCC CTG GAC ACC AGC TCC GTC	I R L P G F H G L R S P L D T S S V	1007				

Fig. 1 (Continued)

3383	3392	3401	3410	3419	3428	
CTC TAT ACT GCC GTG CAG CCC AAT GAG GGT GAC AAC GAC TAT ATC ATC CCC CTG	L Y T A V Q P N E G D N D Y I I P L	1025				
3437	3446	3455	3464	3473	3482	
CCT GAC CCC AAA CCC GAG GTT GCT GAC GAG GGC CCA CTG GAG GGT TCC CCC AGC	P D P K P E V A D E G P L E G S P S	1043				
3491	3500	3509	3518	3527	3536	
CTA GCC AGC TCC ACC CTG AAT GAA GTC AAC ACC TCC TCA ACC ATC TCC TGT GAC	L A S S T L N E V N T S S T I S C D	1061				
3545	3554	3563	3572	3581	3590	
AGC CCC CTG GAG CCC CAG GAC GAA CCA GAG CCA GAG CCC CAG CTT GAG CTC CAG	S P L E P Q D E P E P Q L E L Q	1079				
3599	3608	3617	3626	3635	3644	
GTG GAG CCG GAG CCA GAG CTG GAA CAG TTG CCG GAT TCG GGG TGC CCT GCG CCT	V E P E P E L E Q L P D S G C P A P	1097				
3653	3662	3671	3684	3694	3704	
CGG GCG GAA GCA GAG GAT AGC TTC CTG TAG GGGGCTGGCC CCTACCCCTGC CCTGCCTGAA	R A E A E D S F L .	1106				
3714	3724	3734	3744	3754	3764	3774
GCTCCCCCCC TGCCAGCAC CAGCATCTCC TGGCCTGGCC TGACCGGGCT TCCTGTCAGC CAGGCTGCC						
3784	3794	3804	3814	3824	3834	3844
TTATCAGCTG TCCCCCTCTG GAAGCTTTCT GCTCCTGACG TGTGTGCCCC CAAACCTGG GGCTGGCTTA						
3854	3864	3874	3884	3894	3904	3914
GGAGGCAAGA AAACCTGCAGG GGCCGTGACC AGCCCTCTGC CTCCAGGGAG GCCAACTGAC TCTGAGCCAG						
3924	3934	3944	3954	3964	3974	3984
GGTTCCCCCA GGGAACTCAG TTTTCCCATA TGTAAGATGG GAAAGTTAGG CTTGATGACC CAGAACCTAG						
3994	4004	4014	4024	4034	4044	4054
GATTCTCTCC CTGGCTGACA GGTGGGGAGA CCGAATCCCT CCCTGGGAAG ATTCTTGAG TTACTGAGGT						
4064	4074	4084	4094	4104	4114	4124
GGTAAATTAA CTTTTTCTG TTCAGCCAGC TACCCCTCAA GGAATCATAG CTCTCTCCTC GCACCTTTTA						
4134	4144	4154	4164	4174	4184	4194
TCCACCCAGG AGCTAGGGAA GAGACCCCTAG CCTCCCTGGC TGCTGGCTGA GCTAGGGCCT AGCCTTGAGC						
4204	4214	4224	4234	4244	4254	4264
AGTGTTGCCT CATCCAGAAG AAAGCCAGTC TCCTCCCTAT GATGCCAGTC CCTGCGTCC CTGGCCCCGAG						
4274	4284	4294	4304	4314	4324	4334
CTGGTCTGGG GCCATTAGGC AGCCTAATTG ATGCTGGAGG CTGAGCCAAG TACAGGACAC CCCCAGCCTG						
4344	4354	4364	4374	4384	4394	4404
CAGCCCTTGC CCAGGGCACT TGGAGCACAC GCAGCCATAG CAAGTGCCTG TGTCCCTGTC CTTCAGGGCCC						
4414	4424	4434	4444	4454	4464	4474
ATCAGTCCTG GGGTTTTTC TTTATCACCC TCAGTCTTAA TCCATCCACC AGAGTCTAGA AGGCCAGACG						
4484	4494	4504	4514	4524	4534	4544
GGCCCCGCAT CTGTGATGAG AATGTAAATG TGCCAGTGTG GAGTGGCCAC GTGTGTGTGC CAGTATATGG						

Figure 2

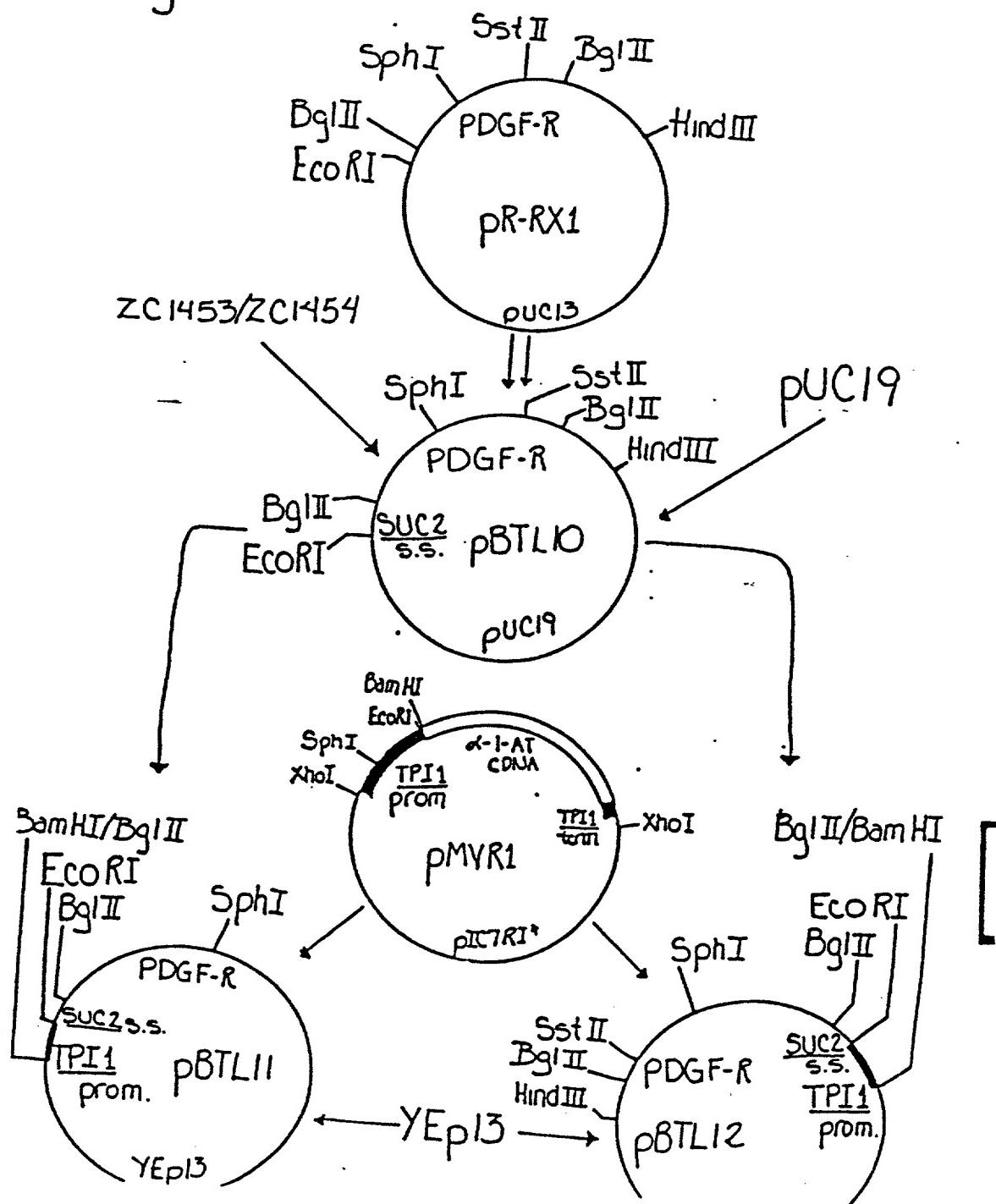


Figure 3

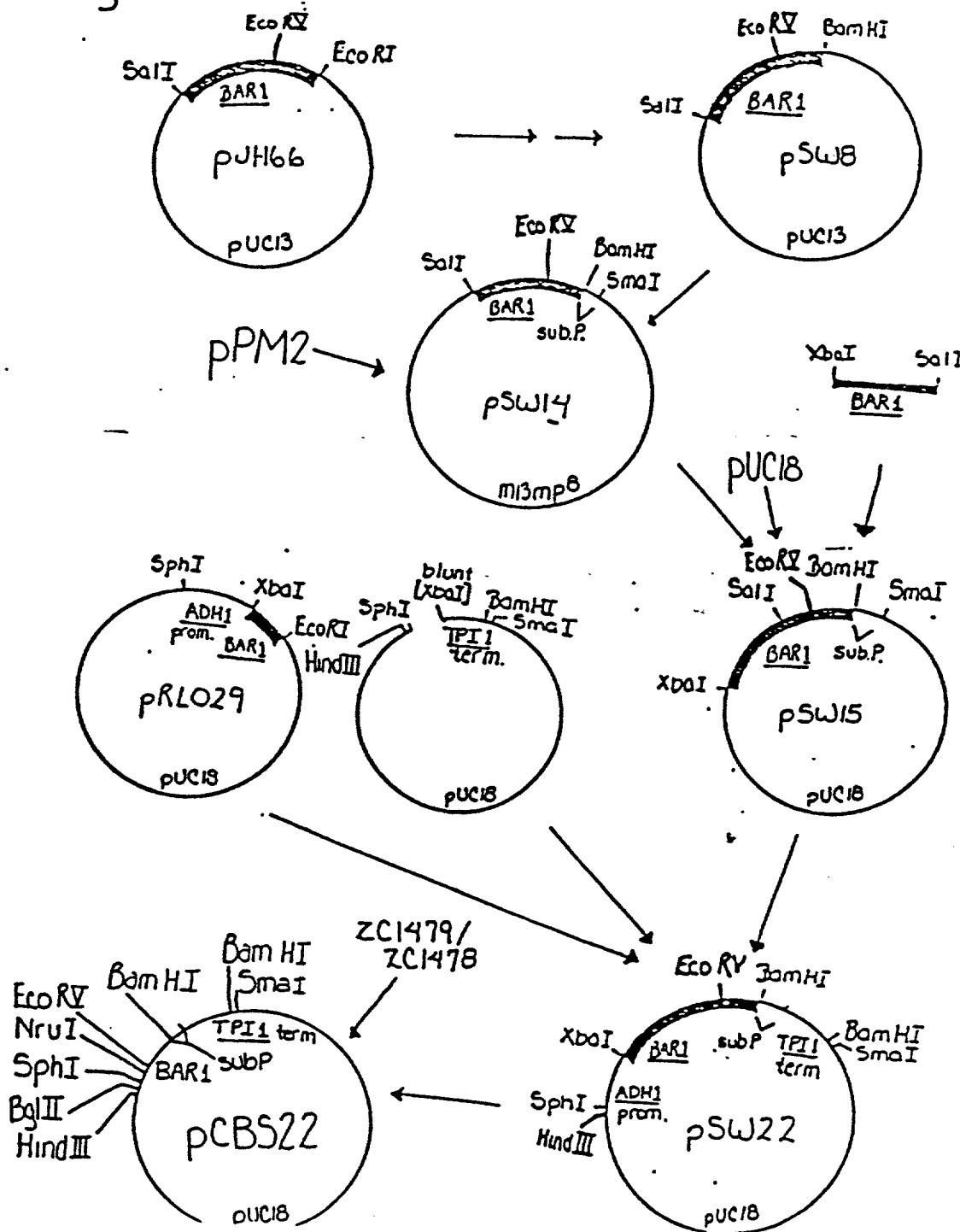
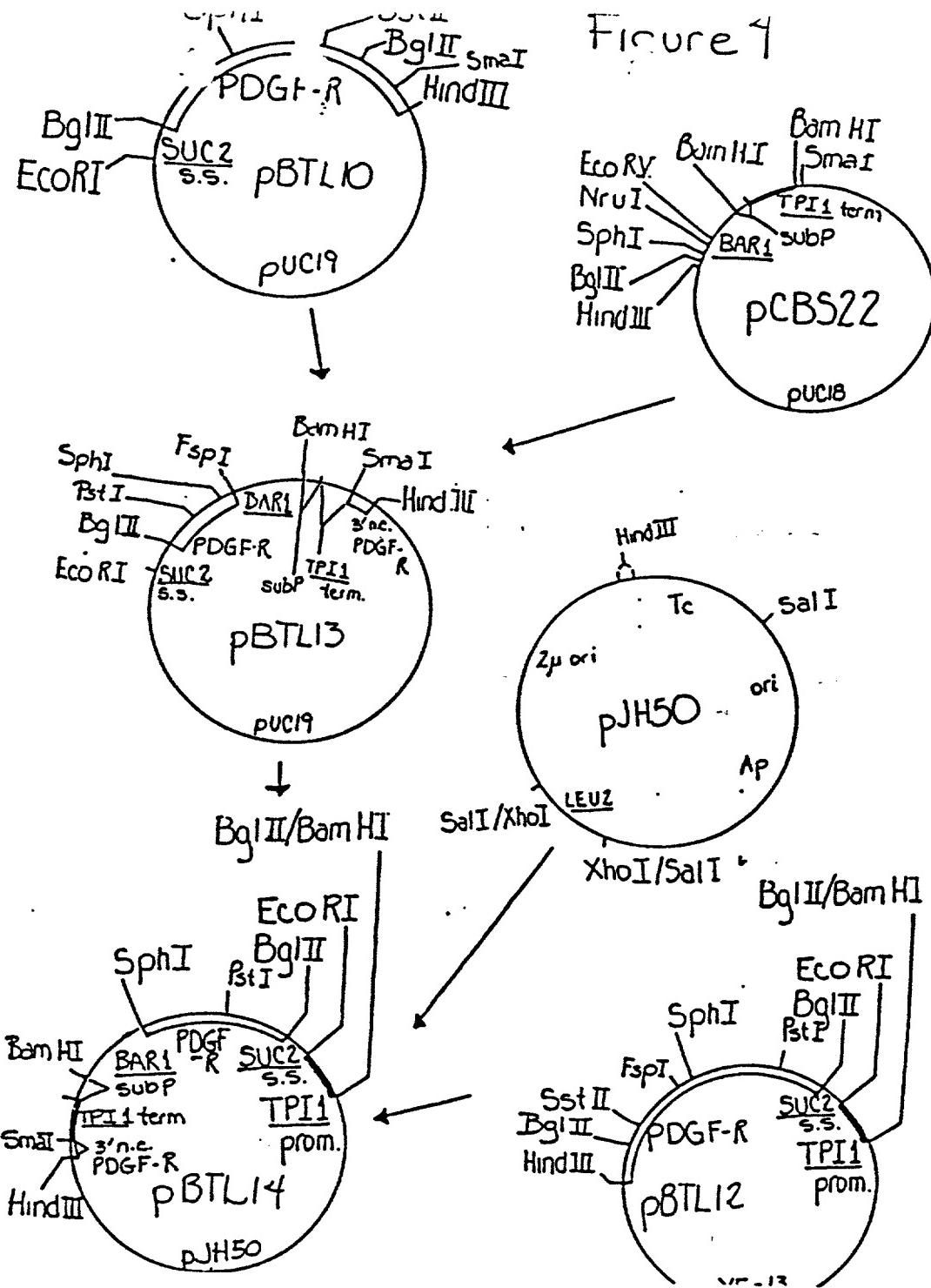
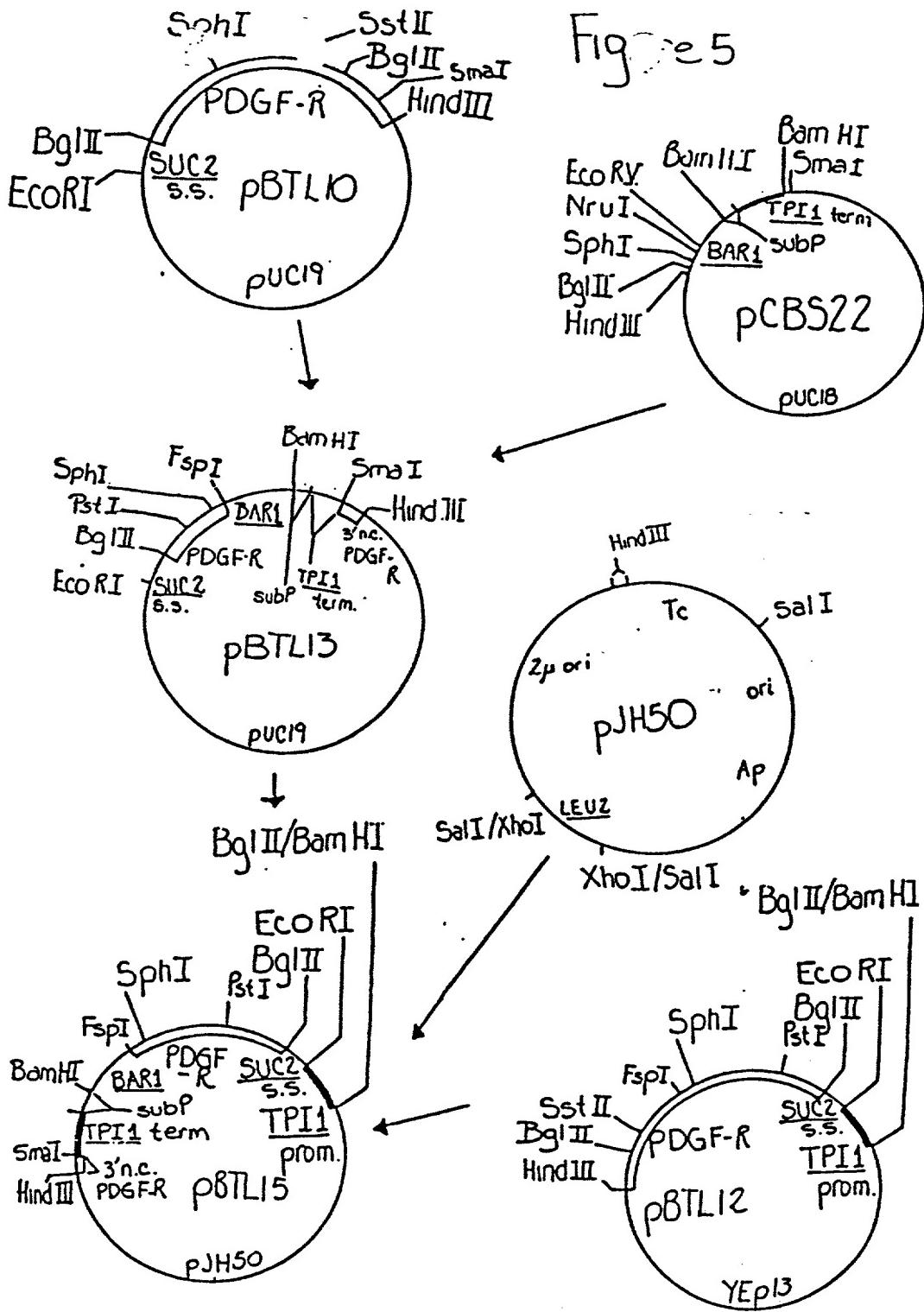


Figure 4





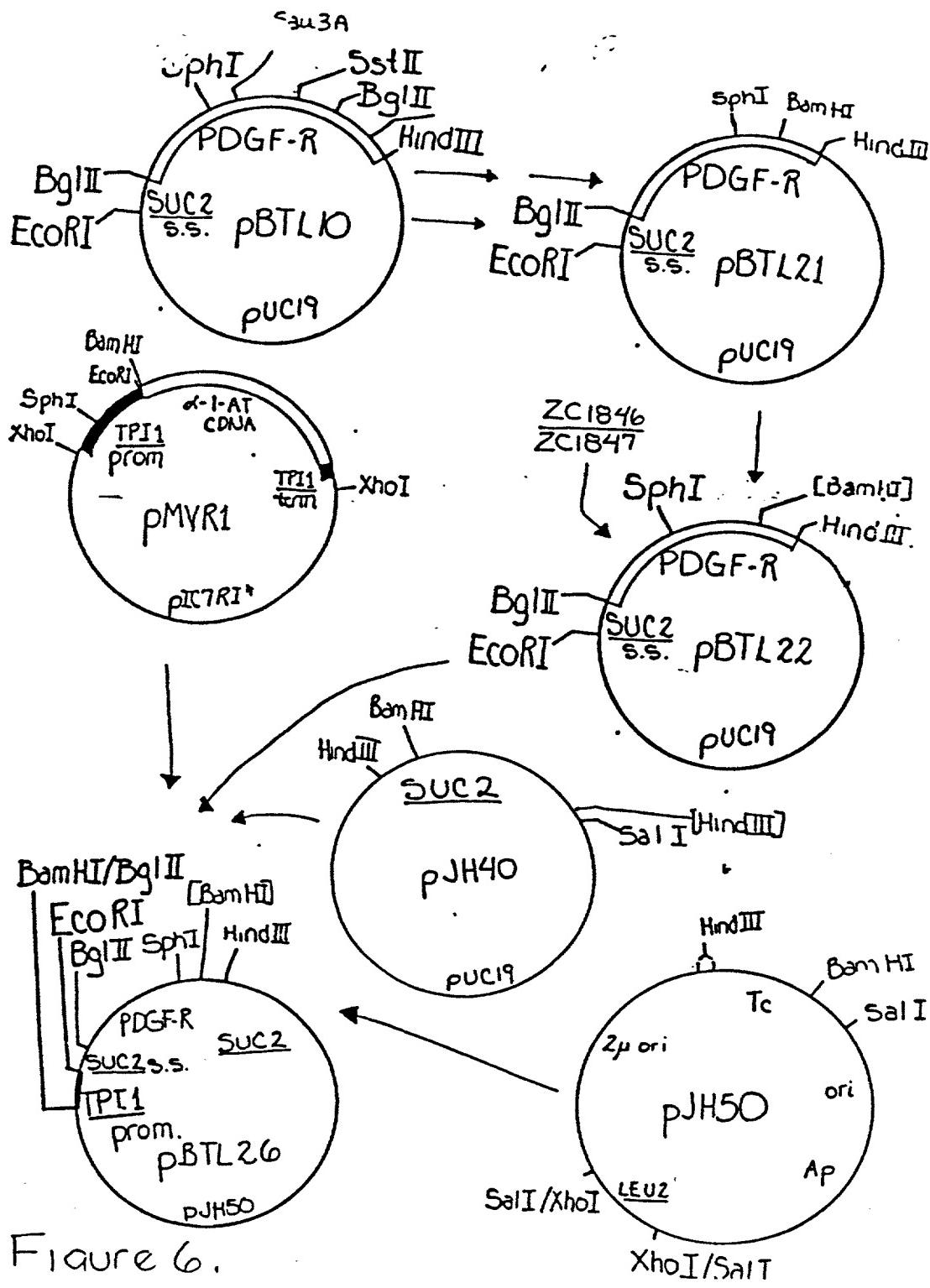


Figure 6.

Figure 7

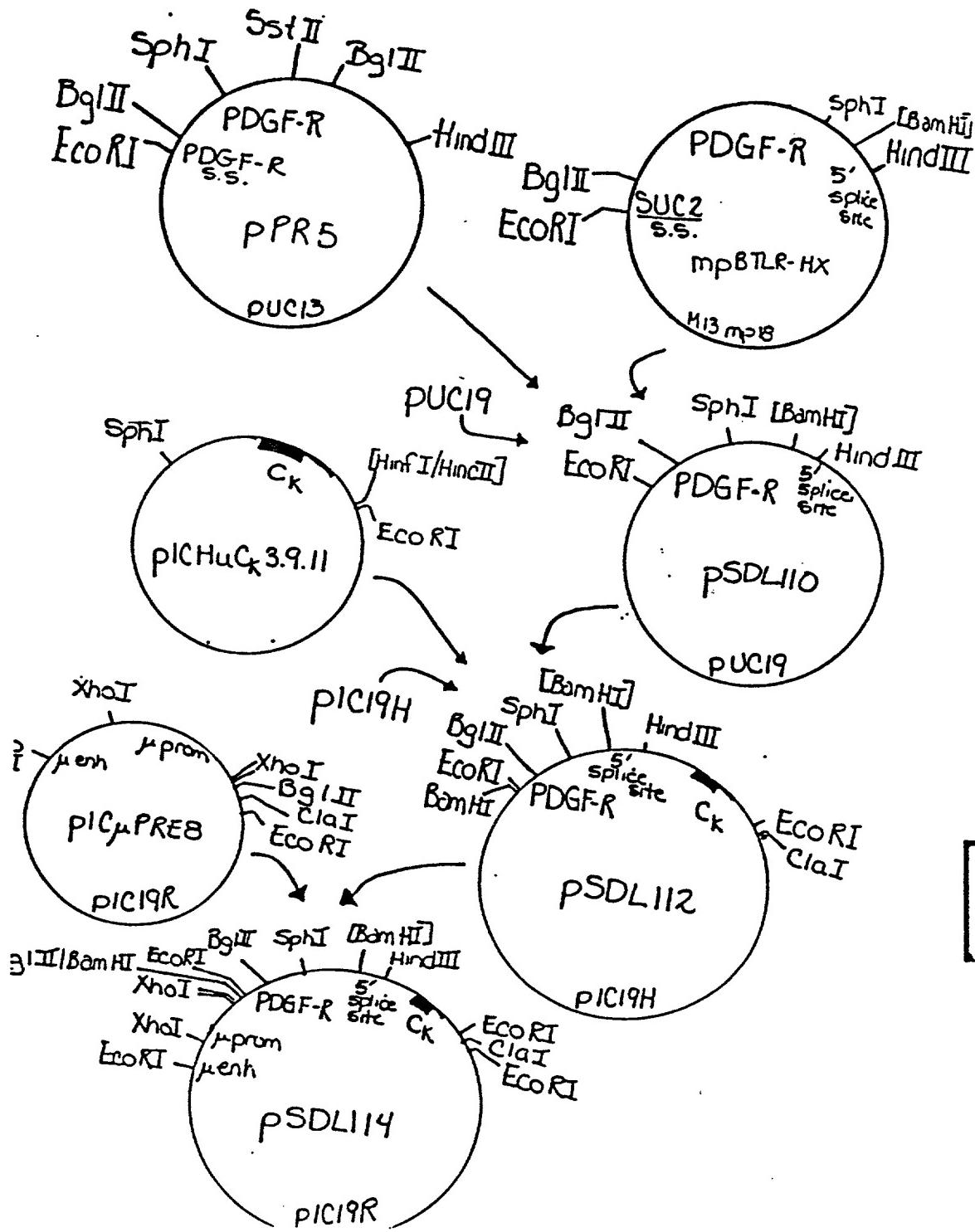
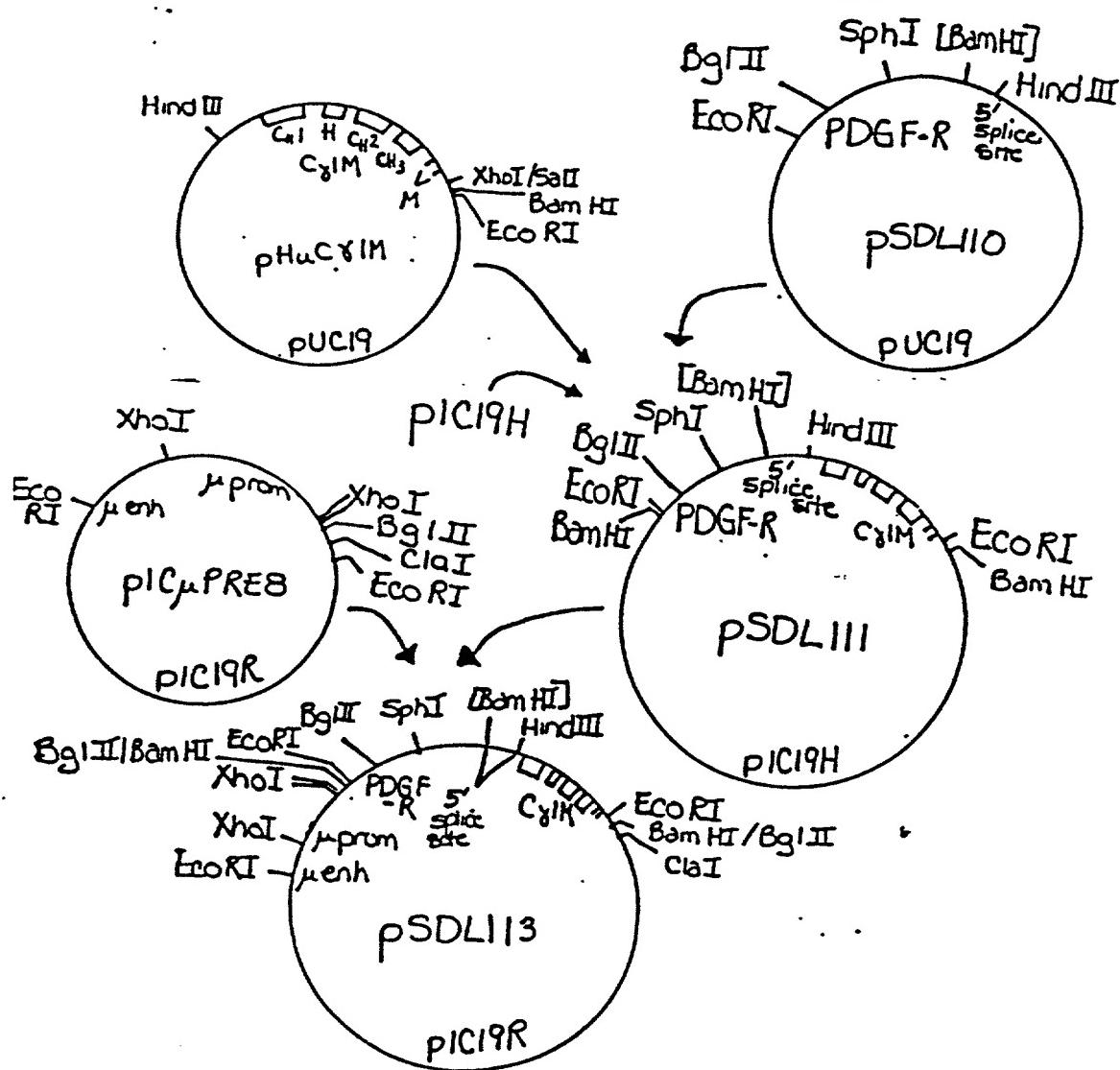


Figure 8



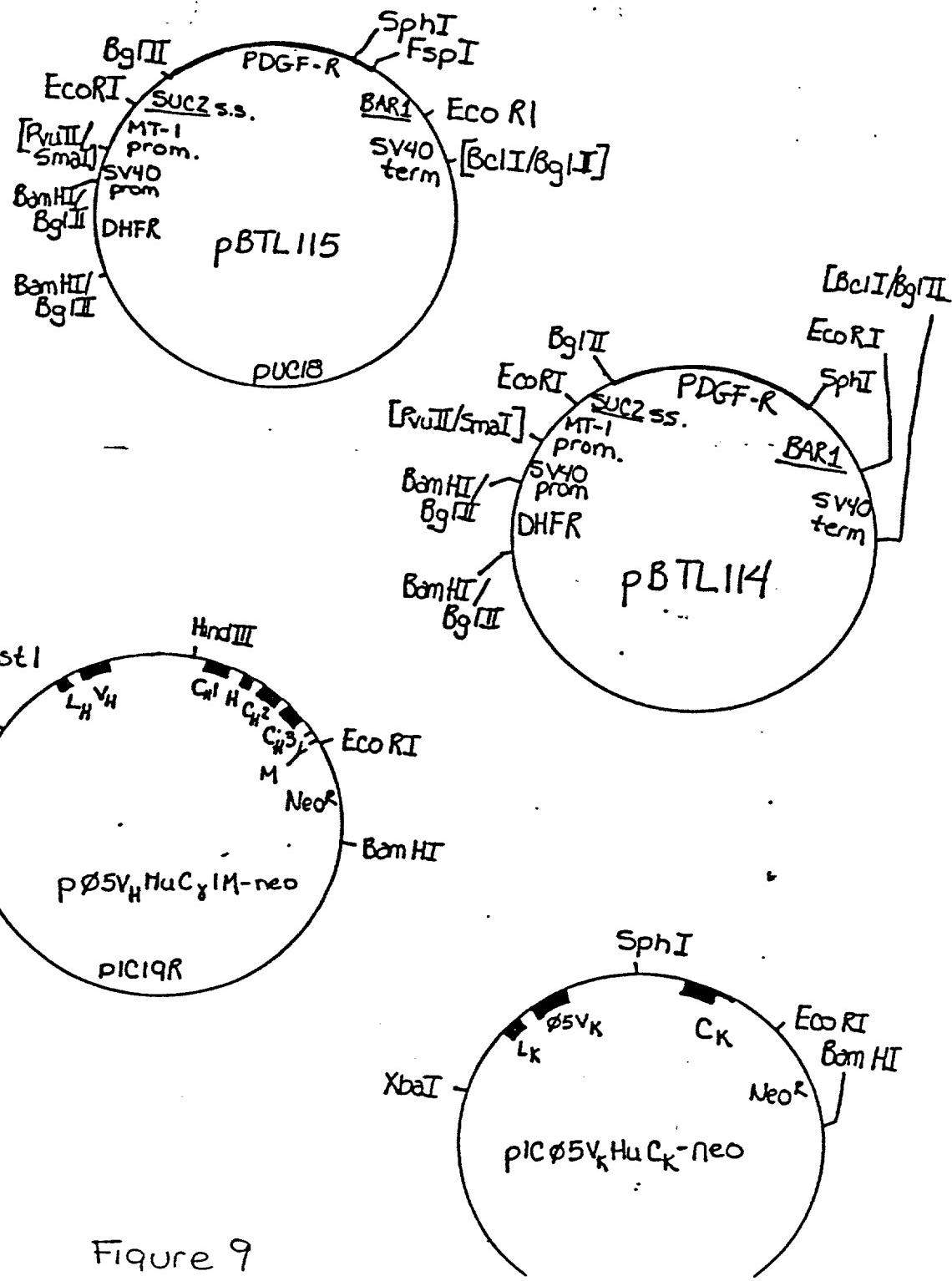


Figure 9

Figure 10

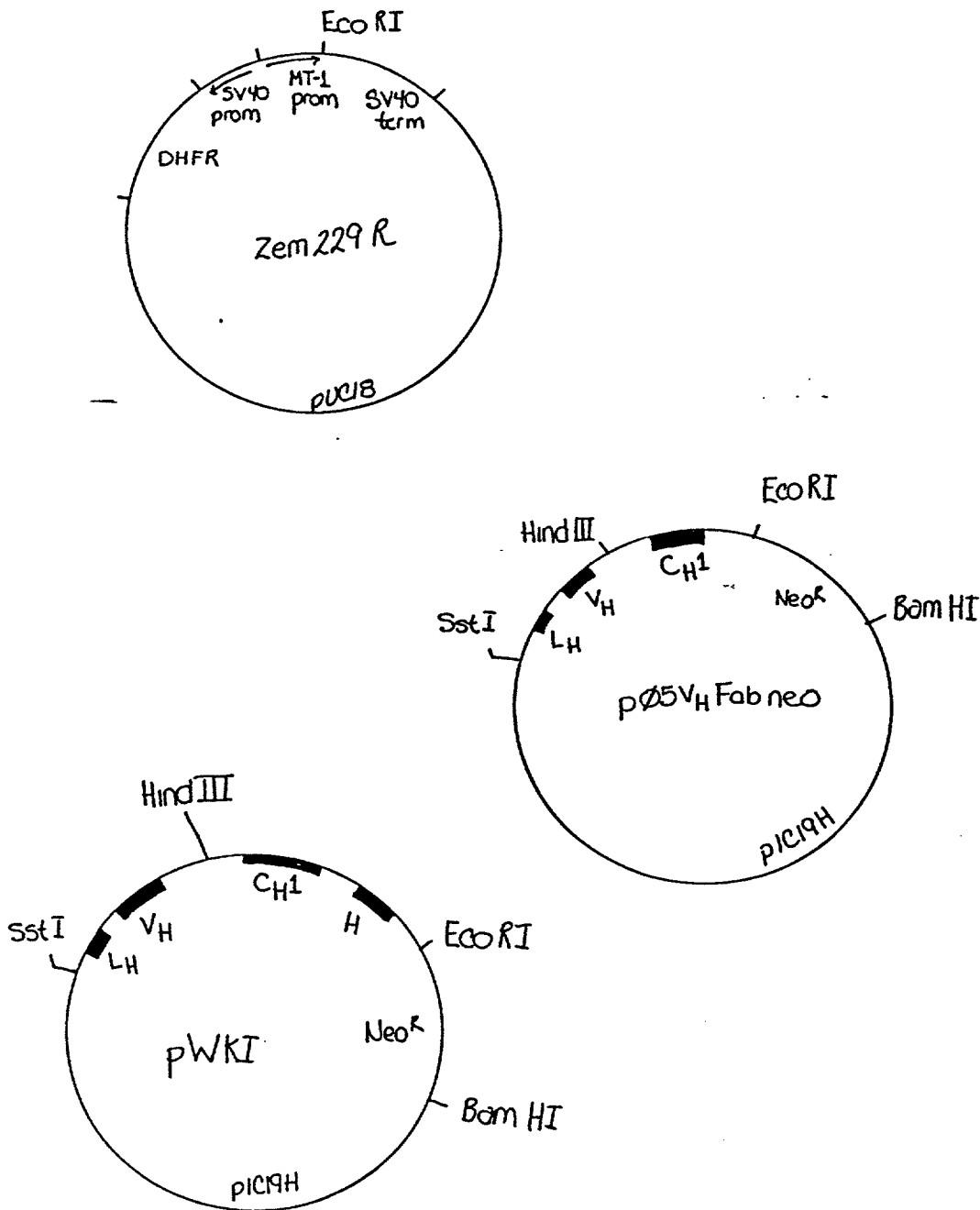


FIGURE 1

1 GCCCTGGGACGGACCGTGGCGGCCAGCGGGACCCGTTTGGGCACGTGGCCAGGCC
70 TCCCTGCACACCCACAGGAAGTACTCCCTTGACCTCGGGAGCTGGACCAAGGTTAACGTTGCTGG
139 TCGAAAAGTGACAATTCTAGGAAAAGAGCTAAAGCCGGATCGGTGACCGAAAAGTTCCCAGAGCTATG
M
1
208 GGGACTTCCCATCCGGCTTCTGGCTTAGGCTGCTCTCACAGGGCTGAGCCTAATCCTCTGCCAG
G T S H P A F L V L G C L L T G L S L I L C Q
277 CTTCATTACCCCTATCCITCAAATGAAAAGCTTGTGAGCTGAATTCTATCCCTTCTCTG
L S L P S I L P N E N E K V V Q L N S S F S L
346 AGATGCTTGGGGAGAGTGAAAGTGAGCTGGCAGTACCCATGTCTGAAGAAGAGAGCTCCGATGTGGAA
R C F G E S E V S W Q Y P M S E E E S S D V E
415 ATCAGAAAATGAAGAAAACAACAGCGGCCCTTTGTGACGGCTTGGAAAGTGAGCAGTGCCTCGCGGCC
I R N E E N N S G L F V T V L E V S S A S A A
484 CACACAGGGTGTACACTTGTCTTACAAACCAACTCAGACAGAAGAGAATGAGCTTGAAAGCAGGCAC
H T G L Y T C Y Y N H T Q T E E N E L E G R H
553 ATTACATCTATGTGCCAGACCCAGATGTAGCCTTGTACCTCTAGGAATGACGGATTATTAAGTCATC
I Y I Y V P D P D V A F V P L G M T D Y L V I
622 GTGGAGGATGATGATTGTGCCATTATAACCTTGTGCAACAATGATCCGAGACTCCTGTAACCTTACAC
V E D D D S A I I P C R T T D P E T P V T L H
691 AACAGTGAGGGGTGGTACCTGCCCTTACCGACAGCAGACAGGGCTTAATGGACCTTCACTGTAGGG
N S E G V V P A S Y D S R Q G F N G T F T V G
760 CCCTATATCTGTGAGGCCACCGTCAAAGGAAAAGAAGTTCCAGACCCATCCCTTAAATGTTATGCTTAA
P Y I C E A T V K G K K F Q T I P F N V Y A L
829 AAAGCAGACATCAGAGCTGGATCTAGAAAATGGAAGCTTAAACCGGTGTATAAGTCAGGGAAACGATT
K A T S E L D L E M E A L K T V Y K S G E T I
898 GTGGTCACCTGTGCTGTTAAACAATGAGGTGGTTGACCTTCAATGGACTTACCCCTGGGACAAGTGAAA
V V T C A V F N N E V V D L Q W T Y P G E V K

Figure 11 continued

967 GCGAAAGGCATCACAACTGGAAAGAAATCAAAGTCCCACATCAAATTGGTGTACACTTTGACGGTC
G K G I T I L E E I K V P S I K L V Y T L T V

1036 CCCGAGGCCACGGTCAAAGACAGTGGAGATTACGAATGTGCTGCCGCCAGGCTACCAGGGAGGTCAA
P E A T V K D S G D Y E C A A R Q A T R E V K

1105 GAAATGAAGAAAGTCACATTTCTGCCATGAGAAAGGTTTCATTGAAATCAAACCCACCTTCAGCCAG
E M K K V T I S V H E K G F I E I K P T F S Q

1174 TTGGAAGCTGTCAACCTGCATGAAGTCAAACATTGTTGAGAGGTGCCCTACCCACCTCCAGG
L E A V N L H E V K H F V V E V R A Y P P P R

1243 ATATCCTGGCTAAAAACAATCTGACTCTGATTGAAAATCTCACTGAGATCACCCTGATGTGGAAAAG
I S W L K N N L T L I E N L T E I T T D V E K

1312 ATTCAAGGAATAAGGTATCGAAGCAAAATTAAAGCTGATCCGTGCTAAGGAAGAACAGTGGCATTAT
I Q E I R Y R S K L K L I R A K E E D S G H Y

1381 ACTATTGTAGCTAAAATGAAGATGCTGTGAAGAGCTATACCTTGAACTGTTAACTCAAGTTCCCTCA
T I V A Q N E D A V K S Y T F E L L T Q V P S

1450 TCCATTCTGGACTTGGTCATGATCACCATGGCTCAACTGGGGACAGACGGTAGGGTGCACAGCTGAA
S I L D L V D D H H G S T G G Q T V R C T A E

1519 GGCACGCCGCTTCTGATATTGAGTGGATGATGCAAGATATAAGAAATGTAATAATGAAACTTCC
G T P L P D I E W M I C K D I K K C N N E T S

1588 TGGACTATTTGCCAACATGCTCAACATCATCACGGAGATCCACTCCGAGACAGGAGTACCGTG
W T I L A N N V S N I I T E I H S R D R S T V

1657 GAGGGCCGTGTGACTTTGCCAACAGTGGAGGAGACCATGCCGTGCGATGCCCTGGCTAAGAATCTCCCT
E G R V T F A K V E E T I A V R C L A K N L L

1726 GGAGCTGAGAACCGAGAGCTGAAGCTGGCTCCACCCCTGCCTCTGAACTCACGGTGGCTGCTGCA
G A E N R E L K L V A P T L R S E L T V A A A

1795 CTCCCTGGCTGTGGTCAATTGTGATCATCTCACTTATTGTCCCTGGTTGTCAATTGGAAACAGAACCG
V L V L L V I V I I S L I V L V V I W K Q K P

Figure II continued

1864 AGGTATGAAATTGGCTGGAGGGTCAATTGAATCAATCAGCCCGGATGGACATGAAATATATTTATGTGGAC
 R Y E I R W R V I E S I S P D G H E Y I Y V D
 1933 CCGATGCAGCTGCCCTATGACTCAAGATGGGAGTTCCAAGAGATGGACTAGTGCTTGGTCGGCTTGG
 P M Q L P Y D S R W E F P R D G L V L G R V L
 2002 GGGTCTGGAGCGTTGGGAAGGTGGTGAAGGAACAGCCTATGGATTAAGCCGGTCCCACCTGTCTG
 G S G A F G K V V E G T A Y G L S R S Q P V H
 2071 AAAGTTGCAGTGAAGATGCTAAAAACCCACGGCCAGATCCAGTGAAAAAACAGCTCTCATGTCTGAAC
 X V A V K M L K P T A R S S E K Q A L M S E L
 2140 AAGATAATGACTCACCTGGGGCACATTTGAACATTGTAAACTTGCTGGGAGCCTGCACCAAGTCAGGC
 K I M T H L G P H L N I V N L L G A C T K S G
 2209 CCCATTTACATCATCACAGAGTATTGCTCTATGGAGATTGGTCAACTATTGGATAAAGAATAGGGAT
 P I Y I I T E Y C F Y G D L V N Y L H K N R D
 2278 AGCTTCTGAGCCACCACCCAGAGAACGCAAAGAAAGAGCTGGATATCTTGGATTGAACCTGCTGAT
 S F L S H H P E K P K K E L D I F G L N P A D
 2347 GAAAGCACACGGAGCTATGTTATTITATCTTGTAAATGGTCAACTACATGGACATGAAACAGGCT
 E S T R S Y V I L S F E N N G D Y H D M K Q A
 2416 GATACTACACAGTATGCCCCATGCTAGAAAAGGAAAGAGGTTCTAAATATTCCGACATCCAGAGATCA
 D T T Q Y V P M L E R K E V S K Y S D I Q R S
 2485 CTCTATGATCGTCCAGCCTCATATAAGAAGAAAATCTATGTTAGACTCAGAAGTCAAAACCTCTTCA
 L Y D R P A S Y K K S M L D S E V K N L L S
 2554 GATGATAACTCAGAAGGCCTTACTTTATTGGATTTGGTCACTTACCTATCAAGTTGGCCGAGGAATG
 D D N S E G L T L D L L S F T Y Q V A R G M
 2623 GAGTTTTGGCTTCAAAAAATTGTGTCCACCGTGATCTGGCTGCTGGCAACGTCCTCTGGCACAGGA
 E F L A S K N C V H R D L A A R N V L L A Q G
 2692 AAAATTGTGAAGATCTGTGACTTTGGCTGGCCAGAGACATCATGCATGATTQGAACATCTGTGAAA
 K I V K I C D F G L A R D I M H D S N Y V S K
 2761 GGCAGTACCTTCTGCCCTGAACTGGATGGCTCTGAGAGCATCTTGACAAACCTCTACACCCACACTG
 G S T F L P V K W M A P E S I F D N L Y T T L

2830 AGTGATGTCGGTCTATGGCATTCTGCTCTGGAGATCTTTCCCTGGTGGCACCCCTACCCGGC
S D V W S Y G I L L W E I F S L G G T P Y P G

2899 ATGATGGTGGATTCTACTTTCTACAATAAGATCAAGAGTGGTACCGGATGCCAAGCTGACCACGCT
M M V D S T F Y N K I K S G Y R H A K P D H A

2968 ACCAGTGAAGTCTACGGAGATCATGGTAAATGCTGGAACAGTGAGCCGGAGAAGAGACCCCTTTAC
T S E V Y E I M V K C W N S E P E K R P S F Y

3037 CACCTGAGTGGAGATTGTGGAGAATCTGCTGCCGGACAATATAAAAAGAGTTATGAAAAAAATTACCTG
H L S E I V E N L L P G Q Y K K S Y E K I H L

3106 GACTTCCTGAAGAGTGACCATCCTGCTGTGGCACGGCATGGTGTGGACTCAGACAATGCATACATTGGT
D F L K S D H P A V A R M R V D S D N A Y I G

3175 GTCACCTACAAAAACGAGGAAGACAAGCTGAAGGACTGGGAGGGTGGTCTGGATGAGCAGAGACTGAGC
V T Y K N E E D K L K D W E G G L D E Q R L S

3244 GCTGACAGTGGCTACATCATTCTGCTGACATTGACCCCTGCTGGAGGAGCTGGCAAC
A D S G Y I I P L P D I D P V P E E E D L G K

3313 AGGAACAGACACAGCTCCAGACCTCTGAAGAGAGTGCCATTGAGACGGGTTCCAGCAGTTCCACCTTC
R N R H S S Q T S E E S A I E T G S S S S T F

3382 ATCAGAGAGAGGAGGACGACCATTAAGACATCGACATGATGGACGACATGGCATAGACTTTAGAC
I K R E D E T I E D I D M M D D I G I D S S D

3451 CTGGTGGAAAGACAGCTTCTGTAACTGGCGATTGAGGGGTTCCACTCTGGGGCACCTCTGG
L V E D S F L
1089

3520 ATCCCCTCAGAAAACCACTTTATTGCAATGGGGAGGTTGAGAGGGAGACTGGTGTGATGTTAAAGAG
3589 AAGTTCCCAGCCAAGGGCTCTGGGGAGCGTCTAAATATGAATGGGATATTGAAATGAACCTT
3658 GTCAGTGTGCTCTTGCATGGCTCAGTAGCATCTCAGGGTGTGAAGTTGGAGATAGATGGATA
3727 AGGGATAATAGGCCACAGAAGGTGAACCTTGTGCTCAAGGACATTGGTGAAGGTCAAACAGACACAA
3796 TTATTAACCTGGCACAGAACCTCAGCATTGTAATTATGTAATTAAACTCTAACCAAGGCTGTGTTAGATTG
3865 TATTAACCTATCTCTGGACTCTGAAGAGACCACTCAATCCATCTGTACTTCCCTTGTAAACCTG
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4003 ACTATAGCATTGCTATCTTTAGTGTAAAGAGATAAGAATAAAAG

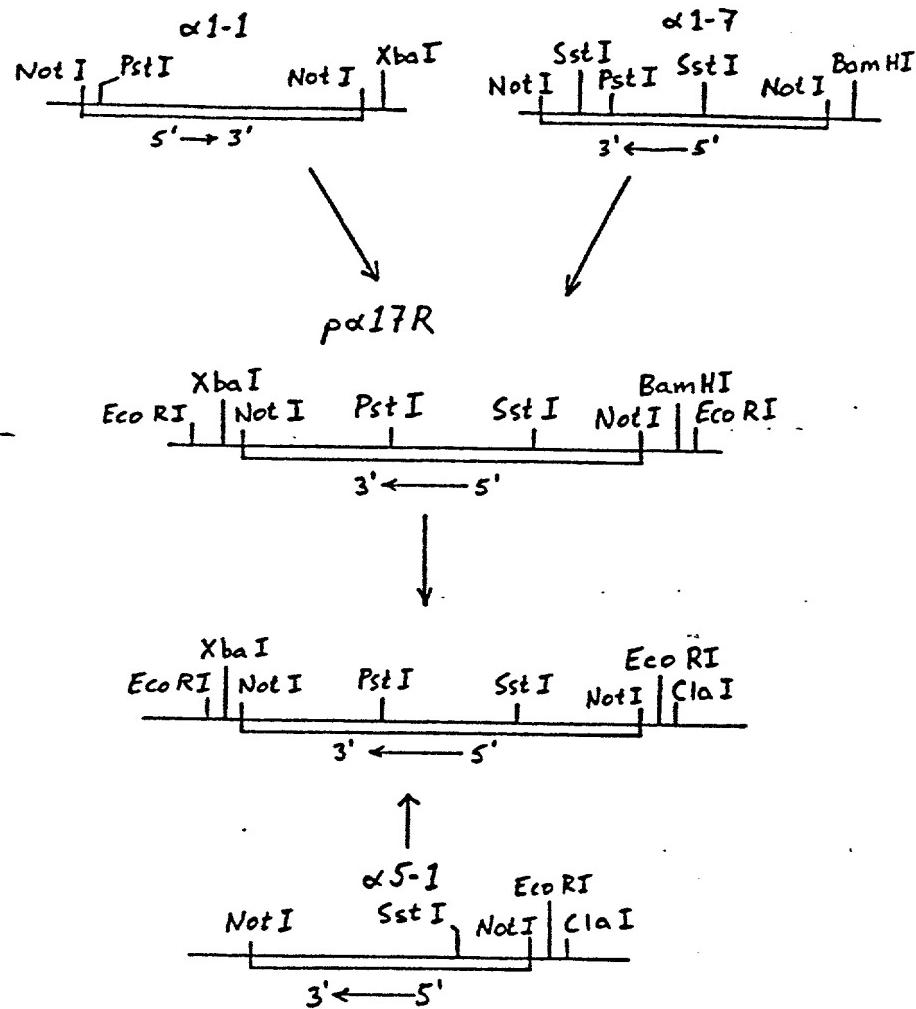


FIGURE 12